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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	**Denise Faustman**	Examiner:	Nolan
Serial No.:	**09/258,682**	Group Art Unit:	1644
Filed:	**February 26, 1999**		
Entitled:	**"Methods for Diagnosing and Treating Autoimmune Disease"**	Conf. No.:	4225

Commissioner for Patents
Washington, D.C. 20231

DECLARATION OF DENISE FAUSTMAN UNDER 37 C.F.R. §1.132

Sir:

I, Denise Faustman M.D., Ph.D., hereby declare that:

1. I received a Ph.D. and M.D. degree from Washington University in 1987. I am currently an Associate Professor of Medicine, Harvard Medical School and Director of the Immunobiology Laboratory at Massachusetts General Hospital. I perform research in the field of Autoimmunity and Transplantation. I have spent the last decade researching the nature of the molecular defect in T-cells that results in the development of autoimmunity. This work led me to discover a novel way to treat spontaneously autoimmune mice, accomplishing for the first time ever, the permanent reversal of established diabetes. My research publications relating to autoimmune diseases include the following:

Penfornis A, Tuomilehto-Wolf E, Faustman DL, Hitman GA.
Analysis of TAP2 polymorphisms in Finnish individuals with type I diabetes.
Hum Immunol. 2002 Jan;63(1):61-70.

Hayashi T, Faustman DL.

Selected contribution: Association of gender-related LMP2 inactivation with autoimmune pathogenesis.
J Appl Physiol. 2001 Dec;91(6):2804-15.

Ryu S, Kodama S, Ryu K, Schoenfeld DA, Faustman DL.
Reversal of established autoimmune diabetes by restoration of endogenous beta cell function.
J Clin Invest. 2001 Jul;108(1):63-72.

Hayashi T, Faustman DL.
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Apoptosis. 2001 Feb-Apr;6(1-2):31-45. Review.

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Arch Immunol Ther Exp (Warsz). 2000;48(5):353-65. Review.

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The role of the proteasome in autoimmunity.
Diabetes Metab Res Rev. 2000 Sep-Oct;16(5):325-37. Review.

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Reply to 'LMP2 expression and proteasome activity in NOD mice'
Nat Med. 2000 Oct;6(10):1065-6. No abstract available.

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Pinies JA, Fu Y, Vazquez JA, Faustman D.
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Diabetes. 1997 Mar;46(3):363-71.

Huang R, Guo J, Li X, Faustman DL.
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Diabetes. 1995 Sep;44(9):1114-20.

Li F, Linan MJ, Stein MC, Faustman DL.
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Science. 1991 Dec 20;254(5039):1756-61.

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Diabetes. 1991 May;40(5):590-7.

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Analysis of T lymphocyte subsets in all stages of diabetes.
J Autoimmun. 1990 Apr;3 Suppl 1:111-6.

Faustman D, Eisenbarth G, Daley J, Breitmeyer J.
Abnormal T-lymphocyte subsets in type I diabetes.
Diabetes. 1989 Nov;38(11):1462-8.

2. I have read the Office Action mailed in the above-referenced patent application on January 2, 2002, and understand that claims have been rejected for alleged lack of enablement and written description. The Office Action states that the specification has "no working examples that the administration of any agent that restores NFκB activity treats any autoimmune disease and that there is no clear guidance as to how the administration of any agents of recited in claim 43 would be administered to intracytosolically increase NFκB activity". The Office Action further states that "since the specification has no working examples demonstrating that restoring NFκB activity can treat autoimmune diseases it would be unpredictable and require an undue amount of experimentation to practice Applicant's claimed invention."

3. Using the Examiner's comments as a guide, I present the following experimental *in vivo* data to demonstrate that "agents" that stimulate the NF-κB pathway, as disclosed both in the present patent application, and in the art available at the time the present application was filed,

"restore NF-κB activity" and treat the autoimmune disease, including autoimmune type I diabetes and, Sjogren's Syndrome (autoimmunity of the salivary and lacrimal glands) in an animal model.

4. Scientific Principles & Theory Underlying the Present Invention

The NOD mouse is a spontaneous model of autoimmunity that mimics in many ways human autoimmunity (Silveira PA. Baxter AG. Autoimmunity. 34(1): 53-64, 2001; Exhibit G; Kikutani and Makino 1992, *Adv. Immunol.* 51: 285; Exhibit H; Bach *C.R. Acad Sci.* 314: 45; Exhibit I). The NOD disease is associated with genes in the MHC class II region, females predominately get the disease and multiple autoimmune diseases occur in the same animal (Research in Immunology 148:301,1997)These traits mimic human disease.

The NOD mouse spontaneously gets type I diabetes, rheumatoid arthritis, Sjogren's syndrome, autoimmune hemolytic anemia, and auto antibodies reminiscent of a lupus-like disease and is thus an accurate animal model for at least five autoimmune diseases (Research in Immunology 148:301,1997) The NOD mouse is most commonly studied in the context of type I diabetes because the late onset is uniformly lethal within months. The autoimmune disease in the NOD mouse is characterized by T cell infiltrates selectively in the organ being destroyed and the ability of splenocytes of diabetic NOD mice to transfer disease to young NOD mice that are still disease free. This demonstrates the NOD autoimmunity is due to a T cell attack on select self-tissues.

The NOD mouse is a highly studied model of spontaneous autoimmune disease (Silveira PA. Baxter AG. Autoimmunity. 34(1): 53-64, 2001; Exhibit G; Kikutani and Makino 1992, *Adv. Immunol.* 51: 285; Exhibit H; Bach *C.R. Acad Sci.* 314: 45; Exhibit I). The disease predominately occurs in the female with an incidence of 80-90% between 20-40 weeks of age (Taconic Farms, Germantown, New York) The disease, especially diabetes, is rarely observed in male NOD mice. Only about 10-15% of male NOD mice ever develop hyperglycemia although the male NOD predominately develops autoimmune Sjogren's Syndrome (Nature Medicine 5, 601,1999)This sexual dimorphism in disease penetrance is similar to diverse human autoimmune diseases, most forms of autoimmunity predominately occur in females. For NOD mice the time frame of disease is that there are almost no signs of autoimmunity detectable in

young animals typically less than 5 weeks of age but by 6-8 weeks of age the organs subject to autoimmunity begin to show diffuse lymphoid accumulations. A trigger of disease appears to occur at this critical age (Journal of Applied Physiol 91: 2804, 2001).

The scientific literature over the past 15 years has repeatedly shown that therapeutic interventions in a **young** NOD mouse will prevent disease. The critical word here is "young". If the NOD mice are 5-8 weeks of age almost anything cures the animals from ever progressing to full-blown disease. These successful interventions, numbering over 100 include diverse viral infection, bacterial infections, any foreign antibody regardless whether it recognizes CD3, CD4, CD8, caramel food coloring, etc. The remarkable treatments appear to stimulate the immune system and cause some sort of uncharacterized immune deviation that is permanent and beneficial to the animal.

Unfortunately, up until recently, there has been no therapy that can reverse, halt or permanently suspend **established** autoimmune disease. Once NOD mice are diabetic, the lethal form of autoimmunity, the diverse curative interventions listed above are ineffective in older animals. The only exception to curing already diabetic NOD mice that has been lethal total body irradiation followed by bone marrow and islet transplant from the same donor. Obviously this is a very impractical therapeutic solution to translate to human disease.

The instant patent application recognizes for the first time that a critical trigger for the initiation of disease at approximately 5-8 weeks of age is the selective <u>down</u>regulation of NF-κB activation/activity. This appears to be lineage restricted and developmentally controlled. Based on this recognition, the present patent application teaches stimulating the NF-κB signalling pathway to restore immune balance. The claimed invention also permits therapies that treat late diabetic disease, a barrier that has never been overcome prior to the instant invention.

Diverse data included in the present patent application (see Examples 1 and 2, particularly Figures 5, 6, and 7) discloses that NF-κB activation is low or faulty in the NOD mouse lymphoid cells. Furthermore we have shown *in vivo* that the cell populations of lymphocytes that possess these defects in NF-κB activation are indeed the cells with autoreactive potential. The experiment was performed by me, as follows: Splenocytes (2×10^7) isolated and pooled from diabetic NOD females were transferred to each of 21 irradiated young (4 to 8 weeks

old) male NOD recipients. All 21 recipients developed severe hyperglycemia within 10 to 25 days, with a mean ± SD of 18.4 ± 3.6 days. They also demonstrated a pancreatic islet pathology, characterized by extensive and pronounced insulitis, that was indistinguishable from that of NOD females after spontaneous onset of disease at 20 to 30 weeks of age. Culture of splenocytes from diabetic NOD females with TNF-α for 24 h before adoptive transfer prevented the development of hyperglycemia in four (80%) of the five young male recipients for at least 40 days after cell injection, consistent with the notion that TNF-α induces induction of the NFkB signaling pathway in disease causing cells and modifies their ability to transfer or cause disease. Examination of pancreatic histology 40 days after cell transfer in the normoglycemic recipients revealed mild or moderate invasive insulitis, but with visible islet structures thus showing the significant reduction in the disease process. Thus, culture of the splenocytes with TNF-α before adoptive transfer appeared to eliminate highly pathogenic cells, thus significantly slowing the disease process (Data submitted for publication, Kodama et al. JCI under review). This experiment shows that NF-κB induction, at least *in vitro*, of pathogenic T cells is capable of significantly eliminating the disease causing potential of the cells. TNF–α is one of many cytokines, including IL-1α and β, IL-2, IL-3, IL-12, IL-15, IL-17, IL-18, LIF, THANK and TNFβ, that activates NF-κB (see for example, Pahl, 1999, *Oncogene* 18: 6853; Exhibit J).

Based on this data, studies were designed to evaluate late stages of diabetic NOD disease and determine if *in vivo* stimulation of the NF-κB signalling pathway by a diversity of known compounds that work through this pathway will slow, halt or alter late NOD disease course. NF-κB is a very widely studied transcription factor and can be specifically induced by select cytokines (Il-1, TNF, INF-λ), select surface crosslinkers/stimulants (LPS, BCG, CFA) through the TNF receptor superfamily (CD95L, CD40, DR3, Fas, OX40, Trail, CD30, 4-1BB, CD27, LtbetaR, RANK, DR6) or through intracellular signalling proteins or inhibitors (TRAF, RIP). For the TNF receptor superfamily the induction of NF-κB is cell specific. At the time the present patent application was filed, over 150 different stimulators of NFκB signaling were known in the art (Pahl H.L. Oncogene (1999) 18, 6853-6866; Exhibit J). The known stimulators of NFkB signaling include proteins, chemicals, and physical and environmental factors.

4. Experiments

Mice: NOD female mice at greater than 20 weeks of age, a time in disease course considered to be late disease or advanced disease were the hosts used for the described treatments. Most NOD hosts for these experiments had at least one blood sugar measurement greater than 250 mg/dl but in general had not yet developed cachexia with blood sugars greater than 400 mg/dl. Untreated NOD mice at this stage after 40 days of follow-up are dead from the untreated hyperglycemia or have severe weight loss and very high blood sugars.

Methods: Treat NOD mice in late stages of disease with either a single dose of compounds that induce NF-κB or chronic courses were monitored for duration of normoglycemia over a 40-day observation period.

Experimental Results

Table 1

Treatments			Normoglycemia (d40)	
Compound	**Dose**	**Duration**	**n (total)**	**%**
A. Adjuvants that induce endogenous NF-κB				
BCG	0.5 ml	1 X	5	0
	0.5 ml	1 X/ wk X 4 wks	6	83%
CFA	.05 ml	1 X	5	0
	.05 ml	1 X/wk X 4 wks	8	75%
LPS	.1 ml	1 X	5	0
	.1 ml	1 X/wk X 4 wks	6	66%
B. Cytokines that induce NF-κB				
TNF-α	2 ug/ml	1 X	6	0
	2 ug/ml	2 X/wk X 4 wks	10	70%
	4 ug/ml	1 X	20	0%

	4 ug/ml	2 X/wk X 4 wks	20	85%
	10 ug/ml	1 X	10	0%
	10 ug/ml	2 X/wk X 4 wks	10	100%
	20 ug/ml	1 X	10	0%
	20 ug/ml	2 X/wk X 4 wks	10	100%
IL-1	2 ug/ml	1 X	9	0%
	2 ug/ml	2 X/wk X 4 wks	10	4%
INF-λ	2 ug/ml	1 X	10	0%
	2 ug/ml	2 X/wk X 4 wks	12	67%

Model: Diabetic NOD mice generally >20 weeks of age, with two consecutive blood sugars >250 mg/dl.

Goal: Treat NOD mice with known compounds that can stimulate the NF-κB signaling pathway.

Conclusions

Using six different agents (BCG, CFA, LPS, TNF-α. IL-1, and INF-λ, as shown in Table 1) that induce the NF-kB signalling pathway *in vivo* in late stage NOD mice, these studies demonstrate the ability to forestall the development of lethal hyperglycemia, a symptom of late stage autoimmunity. The induction of NF-kB in these late stage diabetic animals had to be chronic; single treatments to induce NF-kB uniformly failed. All animals were killed after 40 days and the NOD mice treated with chronic therapy, by the listed interventions, had visible pancreatic islets with moderate or pronounced elimination of invasive insulitis, indicating successful treatment of their autoimmune disease. These successfully treated NOD mice also had salivary glands free of lymphoid infiltrates, a sign that the Sjogren's Syndrome had been partially or fully eliminated. Splencoytes prepared from these NOD mice receiving chronic therapies failed to transfer disease to young NOD mice again confirming the endogenous modification of the disease course (data not shown).

These results therefore demonstrate that the administration of BCG, CFA, LPS, TNF- α. IL-1, and INF- γ, all of which belong to the well known class of agents which stimulate the NFkB pathway, to NOD mice effectively stimulates NFkB signalling and treats end stage autoimmune disease in the NOD animal model.

5. I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that wilful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such wilful false statements may jeopardize the validity of the application or any patent issuing thereon.

6/26/02

Date

Denise Faustman, M.D., Ph.D.

Exhibit A

(Reproduced from Harrison's Online)

Genetics

Evidence in humans that there are susceptibility genes for autoimmunity comes from family studies and especially from studies of twins. Studies in IDDM, rheumatoid

arthritis, multiple sclerosis, and SLE have shown that approximately 15 to 30% of pairs of monozygotic twins show disease concordance, compared with <5% of dizygotic twins. The occurrence of different autoimmune diseases within the same family has suggested that certain susceptibility genes may predispose to a variety of autoimmune diseases. In addition to this evidence from humans, certain inbred mouse strains reproducibly develop specific spontaneous or experimentally induced autoimmune diseases, whereas others do not. These findings have led to an extensive search for genes that determine susceptibility to autoimmune disease.

The most consistent association for susceptibility to autoimmune disease has been with the major histocompatibility complex (MHC). Many human autoimmune diseases are associated with particular HLA alleles (Chap. 306). It has been suggested that the association of MHC genotype with autoimmune disease relates to differences in the ability of different allelic variations of MHC molecules to present autoantigenic peptides to autoreactive T cells. An alternative hypothesis involves the role of MHC alleles in shaping the T cell receptor repertoire during T cell ontongeny in the thymus. Additionally, specific MHC gene products themselves may be the source of peptides that can be recognized by T cells. Cross-reactivity between such MHC peptides and peptides derived from proteins produced by common microbes may trigger autoimmunity by molecular mimicry. However, MHC genotype alone does not determine the development of autoimmunity. Identical twins are far more likely to develop the same autoimmune disease than MHC-identical nontwin siblings, suggesting that genetic factors other than the MHC also affect disease susceptibility. Recent studies of the genetics of IDDM, SLE, and multiple sclerosis in humans and mice have shown that there are several independently segregating disease susceptibility loci in addition to the MHC.

There is evidence that several other genes are important in increasing susceptibility to autoimmune disease. In humans, inherited homozygous deficiency of the early proteins of the classic pathway of complement (C1, C4, or C2) is very strongly associated with the development of SLE. In mice and humans, abnormalities in the genes encoding proteins involved in the regulation of apoptosis, including Fas (CD95) and Fas ligand (CD95 ligand), are strongly associated with the development of autoimmunity. There is also evidence that inherited variation in the level of expression of certain cytokines, such as TNF- or IL-10, may also increase susceptibility to autoimmune disease.

A further important factor in disease susceptibility is the hormonal status of the patient. Many autoimmune diseases show a strong sex bias, which appears in most cases to relate to the hormonal status of women.

Acknowledgements

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Correspondence and requests for materials should be addressed to A.M.S. (e-mail: andrewms@u.washington.edu). The GenBank accession number for NUD19 cDNA and protein sequences is AY026252.

Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease

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Crohn's disease[1,2] and ulcerative colitis, the two main types of chronic inflammatory bowel disease, are multifactorial conditions of unknown aetiology. A susceptibility locus for Crohn's disease has been mapped[3] to chromosome 16. Here we have used a positional-cloning strategy, based on linkage analysis followed by linkage disequilibrium mapping, to identify three independent associations for Crohn's disease: a frameshift variant and two missense variants of *NOD2*, encoding a member of the Apaf-1/Ced-4 superfamily of apoptosis regulators that is expressed in monocytes. These *NOD2* variants alter the structure of either the leucine-rich repeat domain of the protein or the adjacent region. NOD2 activates nuclear factor NF-kB; this activating function is regulated by the carboxy-terminal leucine-rich repeat domain, which has an inhibitory role and also acts as an intracellular receptor for components of microbial pathogens. These observations suggest that the *NOD2* gene product confers susceptibility to Crohn's disease by altering the recognition of these components and/or by over-activating NF-kB in monocytes, thus documenting a molecular model for the pathogenic mechanism of Crohn's disease that can now be further investigated.

Crohn's disease (CD; MIM 266600) occurs primarily in young adults, with an estimated prevalence of 1 in 1,000 in western countries[1]. Its incidence has increased markedly over the past half century, arguing for the involvement of recent, unidentified, environmental factors[2]. Familial aggregation of the disease suggests that genetic factors may also be involved—an hypothesis that was substantiated in 1996 by the discovery of a susceptibility locus for CD, *IBD1*, on chromosome 16 (ref. 3). Identification of the exact nature of the genetic changes that are implicated in CD susceptibility would provide a specific approach to understanding this common disorder.

Because candidate genes previously localized on chromosome 16 failed to show an association with CD[4,5], we refined the localization of the *IBD1* susceptibility locus by typing 26 microsatellite markers spaced at an average distance of 1 cM in the pericentromeric region

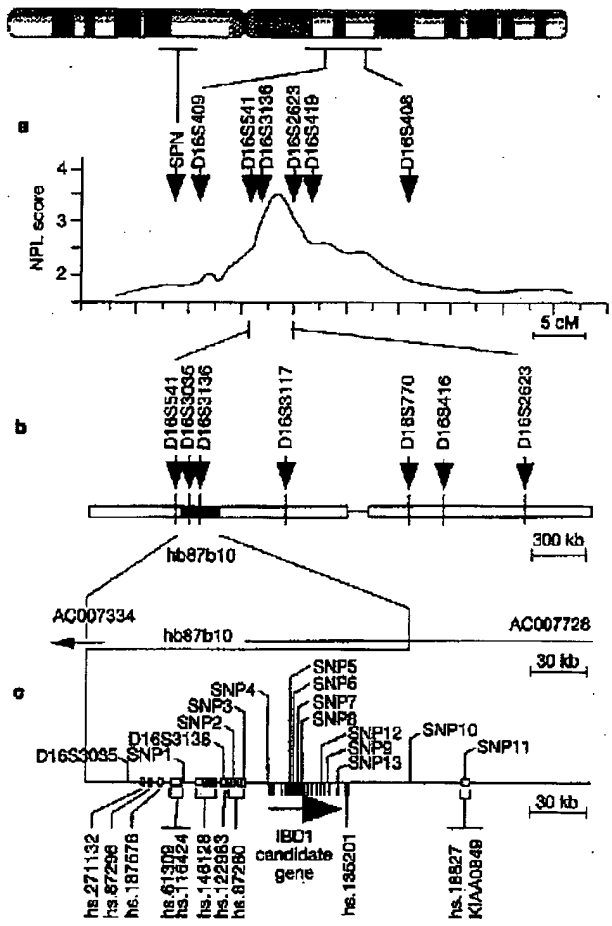

Figure 1 Strategy used to identify the *IBD1* locus. a, Profile for the multipoint non-parametric linkage (NPL) scores. Approximate cytogenetic localizations are shown for selected microsatellite markers used in the study[3] that localized *IBD1* to the pericentromeric region of chromosome 16. Subsequent linkage analyses were focused on the region between SPN and D16S408. The highest NPL score (maximum NPL score, 3.49; $P = 2.37 \times 10^{-4}$) was in the region between markers D16S541 and D16S2623 (ref. 6). b, Physical map of the *IBD1* region[6]. White and black boxes correspond to the two BAC contigs and BAC clone hb87b10, respectively. Five yeast artificial chromosomes (YACs) bridge a gap of ~100 kb between these two contigs. The position on the physical map of the microsatellite markers used in the linkage analysis is indicated. Distance between D16S541 and D16S2623 is ~2 Mb. c, Representation of the sequenced region containing the *IBD1* candidate gene. Unigene clusters and 11 exons of the *IBD1* candidate gene are indicated by white and black boxes, respectively. Bold horizontal arrow indicates direction of transcription. Positions of SNP 1–13, D16S3035 and D16S3136, which were typed in 235 CD families for linkage disequilibrium studies, are shown.

of chromosome 16. Model-free linkage analyses performed on 77 multiplex families indicated that the probability was 0.7 for the location of the susceptibility locus between D16S541 and D16S2623 (Fig. 1a). We constructed bacterial artificial chromosome (BAC) contigs spanning this region (Fig. 1b), which supported linkage disequilibrium mapping[7]. The transmission disequilibrium test[8], performed on a single trio from each of 108 (77 multiplex and 31 simplex) families, showed a borderline-significant association ($P < 0.05$) between the disease phenotype and the 207-base-pair (bp) allele of D16S3136. This observation was replicated with another set of 76 families, although with a different allele (the 205-bp allele; $P < 0.01$). These two observations may be due to type-one errors. Alternatively, they may reflect true association in two sets of families drawn from genetically different populations.

The latter hypothesis led to the following strategy: a 164-kb BAC clone (hb87b10) from the CEPH-BAC library containing D16S3136 was sequenced completely (EMBL accession number AJ303140). A public database search extended the sequence of the corresponding region to 260 kb but did not identify characterized genes, with the exception of KIAA0849, which codes for a ubiquitin C-terminal hydrolase homologue in *Caenorhabditis elegans*. However, analysis by GRAIL and an expressed sequence tag (EST) homology search identified many putatively transcribed regions (Fig. 1c).

Eleven single-nucleotide polymorphisms (SNP 1–11) selected from these regions were genotyped, together with microsatellite markers D16S3035 and D16S3136, in a total of 235 available CD families (Table 1). Strong linkage disequilibrium was observed among most markers (data not shown). Several SNPs showed significant association with CD by the pedigree disequilibrium test[8] (PDT), confirming the existence of linkage disequilibrium, with the disease locus over the investigated region (specially SNP 2, nominal P value 0.00002; Table 1).

These observations prompted the characterization of neighbouring Unigene clusters (Fig. 1c). Eleven overlapping clones, isolated from a human leukocyte complementary DNA library, extended Unigene cluster hs13520 and identified 11 exons of a single gene. The previously identified SNP 5–8 were contained in exon 3 of this gene and shown to be non-synonymous variants. To find additional disease-related variants, all exons of this gene were sequenced in 50 unrelated CD patients—each a member of an affected sibling pair identical by descent for both chromosome 16 homologous regions. Two additional non-synonymous SNPs (SNP 12 and 13), with rare-allele frequencies greater than 0.03, were identified and subsequently used to type the 235 CD families.

The PDT was most significant for SNP 13 ($P = 6 \times 10^{-6}$). Families were divided into two groups: those with at least one member carrying the rare allele of SNP 13 and those without this allele. The latter group of families failed to show association between CD and SNP 4–6, and showed considerable decrease in the significance of the SNP 2 association. This result indicates that the associations of these four loci with CD were not independent of SNP 13 (Table 1). In contrast, significance of the CD associations with SNP 8 and 12 decreased modestly in these families, indicating a minimal contribution of the rare SNP 13 allele to these associations.

The 8 intragenic SNPs that were initially identified defined 41 different haplotypes. Three of these revealed preferential transmission to affected individuals (Table 2). These three haplotypes each contain one rare allele of SNP 8, 12 or 13 in a context of a common background. Notably, the haplotype defined by the same background and by the absence of these rare alleles did not show such transmission distortion (Table 2). Furthermore, the rare alleles of SNP 8, 12 and 13 were never found on the same haplotype, indicating independent association of CD susceptibility with each of three non-synonymous variants of a same gene.

As a result of these associations, the allele frequencies of SNP 8, 12 and 13 differed in the group of CD patients as compared with controls (Table 3). Average risks for CD, computed for genotypes containing zero, one or two variants (Table 3), revealed a gene-

Table 1 Linkage disequilibrium analyses at the *IBD1* locus

Marker name	Distance to next marker (bp)	Rare allele frequency in CD family founders*	Nominal *P*-values for the PDT in CD families†			Marker location/structural features‡
			All ($N = 235$)	SNP 13+ ($N = 65$)	SNP 13– ($N = 170$)	
D16S3035	29,389	Microsatellite marker	NS	0.005	NS	
SNP1	19,434	0.34	NS	NS	NS	End of BAC hb133D1
D16S3136	3,273	Microsatellite marker	NS	0.002	NS	Exon of hs.122963
SNP2	5,459	0.25	0.00002	0.00006	0.02	Exon of hs.87280
SNP3	15,076	0.35	NS	0.005	NS	Exon of hs.87280
SNP4	14,396	0.40	0.0008	0.000003	NS	Grail putative exon
SNP5	576	0.39	0.0001	0.00001	NS	*IBD1* exon 3 721C>T P241S
SNP6	385	0.38	0.0001	0.000006	NS	*IBD1* exon 3 1296C>T R432R
SNP7	344	0.35	NS	0.0003	NS	*IBD1* exon 3 1680T>T R432R
SNP8	10,593	0.10	0.001	0.03	0.009	*IBD1* exon 3 2023C>T R676W
SNP12	2,727	0.03	0.003	NS	0.0041	*IBD1* exon 7 2641G>C G1861R
SNP9	4,510	0.37	0.01	0.00003	NS	*IBD1* intron 8 IVS8+133delAinsCT
SNP13	35,121	0.07	0.000006	0.000006	–	*IBD1* exon 10 2936insG 980fs981X (frame shift)
SNP10	28,592	0.37	0.05	0.002	NS	End of BAC hb27G11
SNP11		0.09	NS	NS	NS	Exon 9 of KIAA0849 1058G>T R352D

*Microsatellite markers are ordered from centromere to telomere. The nomenclature used does not take into account an alternative NOD2 exon located upstream of IBD1 exon 1 (ref. 14). NS, not significant.
†Allele frequencies computed for the 599 CD family founders.
‡Nominal *P*-values of pedigree disequilibrium test (PDT) are reported for all families and the subgroups of families with or without the rare allele of SNP 13.
§When relevant, its effect on the coding sequence are indicated.

dosage effect. The major increase in risk associated with two variant alleles confirms the recessive nature of CD susceptibility, which was suggested by previous segregation analysis[9–12] and linkage studies[3]. It may also contribute to explain the unusual precision of the affected sibling-pair analysis in mapping the susceptibility gene.

The rare allele of SNP 8 was associated positively with the 205-bp allele of D16S3136 and negatively with the 207-bp allele. The inverse association was noted for the rare alleles of SNP 12 and 13, thus providing a rationale for the initial observations made with this microsatellite marker (data not shown). Genotype frequencies were comparable in CD patients originating from uniquely and multiply affected kindred—an observation compatible with the close clinical similarity of the sporadic and familial diseases[12]. The observed linkage of CD to chromosome 16 could not be entirely explained by the present associations, because GeneHunter analysis of 85 multiplex families without SNP 8, 12 and 13 revealed a component of linkage (nonparametric lod score (NPL) 1.6, pointwise significance $P < 0.02$). Thus, other variants of this gene or additional genes on chromosome 16 may be involved in CD susceptibility.

Genotyping of 167 patients with ulcerative colitis revealed genotype frequencies comparable to those of controls, indicating that these SNPs were not associated with susceptibility to ulcerative colitis—an observation in agreement with its lack of linkage to the *IBD1* locus[13].

The candidate *IBD1* gene has high expression in leukocytes, but low or no expression in the other investigated tissues, including normal colon and small intestine (results not shown). It encodes a 1,013-amino-acid protein that is identical to NOD2—a member of the CED4/APAF1 superfamily of apoptosis regulators[14]. From its amino terminus to its carboxy terminus, NOD2 is composed of two caspase recruitment domains (CARD), a nucleotide-binding domain (NBD), and a LRR region (Fig. 2b). The LRR domain of NOD2 has binding activity for bacterial lipopolysaccharides[15] (LPS) and its deletion stimulates the NF-κB pathway[16–18].

The rare allele of SNP 13 corresponds to a 1-bp insertion in exon 10 (980fs) predicted to truncate NOD2 in the LRR region. Those of SNP 8 and 12 cause non-conservative substitutions in the LRR domain (G881R) and in the proximal adjacent region (R675W), respectively (Fig. 2a). Systematic sequencing of the coding sequence of NOD2 revealed additional very rare missense variants, which together were observed in 5% of controls and 4% of patients with ulcerative colitis. This percentage rose to 17% for CD patients, where the most frequent variants tended to cluster in the LRR and its adjacent regions (Fig. 2b). This excess suggests that, in addition to SNP 8, 12 and 13, more variants in this part of the NOD2 protein may be associated with CD susceptibility. Thus, the LRR domain of CD-associated variants is likely to be impaired, possibly to various degrees, in its recognition of microbial components and/or in the physiological inhibition of NOD2 dimerization, thus resulting in the inappropriate activation of NF-κB in monocytes.

Much evidence supports bacteria-induced NF-κB disregulation in CD. First, susceptibility to spontaneous inflammatory bowel

Table 2 Transmission disequilibrium of *NOD2* haplotypes in CD families

SNP no.	Haplotype								Transmitted	Non-transmitted	PDT (*P*-value)
	4	5	6	7	8	12	9	13			
SNP 8	1	1	1	1	2	1	1	1	4	1	
	2	1	1	1	2	1	1	1	2	0	
	2	**1**	**1**	**1**	**2**	**1**	**2**	**1**	82	48	0.001
	1	1	1	1	2	1	2	1	3	4	
	2	1	2	1	2	1	2	1	7	0	
	2	2	2	1	2	1	2	1	0	1	
SNP 12	1	2	2	2	1	2	1	1	1	3	
	2	1	2	1	1	2	1	1	0	1	
	2	**1**	**1**	**1**	**1**	**2**	**2**	**1**	38	13	0.0005
	1	1	1	1	1	2	2	1	3	2	
	2	2	1	1	1	2	2	1	1	1	
	2	2	2	1	1	2	2	1	2	2	
	2	2	2	2	1	2	2	1	3	1	
	1	2	2	2	1	2	2	1	1	0	
	1	1	1	2	1	2	2	1	1	0	
SNP 13	2	1	1	1	1	1	1	2	2	1	
	1	1	1	1	1	1	1	2	0	1	
	2	**1**	**1**	**1**	**1**	**1**	**2**	**2**	83	22	0.0000002
	1	1	1	1	1	1	2	2	2	0	
	1	2	2	1	1	1	2	2	1	0	
	2	2	2	2	1	1	2	2	0	1	
None*	1	2	2	2	1	1	1	1	116	141	NS
	2	2	2	2	1	1	1	1	2	4	
	1	1	2	2	1	1	1	1	0	0	
	1	2	1	2	1	1	1	1	0	2	
	1	1	1	2	1	1	1	1	1	0	
	2	1	1	2	1	1	1	1	1	1	
	1	2	2	1	1	1	1	1	9	19	NS
	1	1	1	1	1	1	1	1	4	7	
	2	1	1	1	1	1	1	1	0	0	
	2	2	2	1	1	1	1	1	2	0	
	2	2	2	2	1	1	2	1	7	4	
	1	2	2	2	1	1	2	1	9	7	
	2	1	2	1	1	1	2	1	0	1	
	1	1	2	1	1	1	2	1	1	1	
	1	2	2	1	1	1	2	1	94	116	NS
	2	2	2	1	1	1	2	1	20	16	NS
	2	**1**	**1**	**1**	**1**	**1**	**2**	**1**	70	78	NS
	1	1	1	1	1	1	2	1	11	7	
	2	2	1	1	1	1	2	1	0	1	
	1	2	1	1	1	1	2	1	3	9	

All haplotypes defined by typing 8 intragenic SNPs of *IBD1* in 235 CD families are indicated. They are classified according to the presence of a rare allele of SNP 8, SNP 12 or SNP 13. The number of transmitted or non-transmitted haplotypes from heterozygous parents to their affected offspring is shown. The statistic used to evaluate transmission distortion is PDT[6]. Haplotypes shown in bold are discussed in the text.

*Includes all haplotypes where none of the rare alleles of SNP8, 12 or 13 are present.

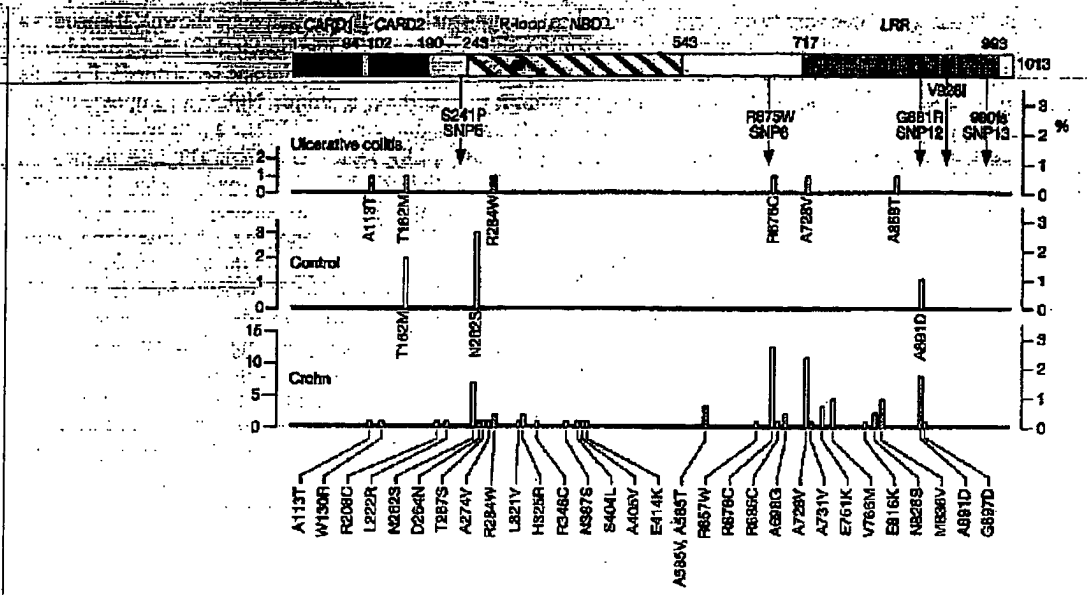

Figure 2 Representation of the IBD1/NOD2 protein variants. The translation product deduced from the cDNA sequence of the candidate *IBD1* gene is identical to that of *NOD2* (ref. 14). The polypeptide contains two caspase recruitment domains (CARD), a nucleotide-binding domain (NBD) and ten 27-amino-acid, leucine-rich repeats (LRRs). Black circle indicates the consensus sequence of the ATP/GTP-binding site motif A (P-loop) of the NBD. The sequence changes encoded by the three main variants associated with CD are SNP 8 (R675W), SNP 12 (G881R) and SNP 13 (980 frameshift). This frameshift changes a leucine to a proline at position 980, and is immediately followed by a stop codon. SNP 5 is described in Table 1. The allele frequencies of the V928I polymorphism were not significantly different (0.92:0.08) in the three groups, and the corresponding genotypes were in Hardy–Weinberg equilibrium. The positions of the rarer missense variants, observed in 457 CD patients, 159 ulcerative colitis patients and 103 unaffected unrelated individuals, are indicated for these groups. Left scale indicates the number of each identified variant in the investigated groups; right scale measures the mutation frequency.

disease (IBD) in mice has been associated with mutations in Toll-like receptor 4 (TLR4)—a member of a family of NF-κB activators that is known to bind LPS through its LRR domain[19,20]. Second, antibiotic therapy causes transient improvement of CD patients, supporting the hypothesis that enteric bacteria may have an aetiological role in CD[21]. Third, NF-κB has a pivotal role in IBD and is activated in mononuclear cells of the intestinal lamina propria in CD[22]. Last, CD treatment is based on the use of sulphasalazine and glucocorticoids—two known NF-κB inhibitors[23,24].

Table 3 Allele and genotype frequencies of the three variants associated with CD

Frequencies of the three rare variant alleles

	Number of chromosomes	SNP 8	SNP 12	SNP 13	Total
Unaffected	206	0.04	0.01	0.02	0.07
UC patients	318	0.03	0.00	0.01	0.05
CD patients	936	0.11	0.06	0.12	0.29

Distribution of variant genotypes and associated risks

	Genotype			
	No variant	Simple heterozygous	Homozygous	Compound heterozygous
Distribution				
Unaffected	88	16	0	0
UC patients	145	13	1	0
CD patients	267	133	28	40
Risk for CD				
Relative risk	1	3	38	44
Absolute risk	7×10^{-4}	2×10^{-3}	3×10^{-2}	3×10^{-2}

Genotypes of patients: simple heterozygous, presence of a single rare variant; homozygous, presence of the same variant on both chromosomes 16; compound heterozygous, presence of two different variants; and no variant. Risk of CD for each genotype is computed assuming a prevalence of one per 1,000 and Hardy–Weinberg equilibrium for these markers in the general population. The three rare variants of SNP 8, 12 and 13 were never observed on the same haplotype. UC, ulcerative colitis.

Genetic susceptibility to CD is not limited to chromosome 16 and at least five additional loci have been implicated[25–29]. The recognition of a transduction pathway that, when disregulated, contributes to the pathogenesis of CD will accelerate the discovery of additional susceptibility genes. It will also contribute to the identification of associated environmental factors and focus the search for specific therapies. □

Methods

Families, microsatellite markers and contig construction

A total of 235 CD families (117 simplex nuclear families, 96 multiplex nuclear families, and 22 extended pedigrees, corresponding to a total of 179 CD patients and 261 unaffected relatives) was progressively recruited according to published diagnostic criteria[30]. In addition, 100 multiplex and 59 simplex ulcerative colitis families were recruited from the same hospitals. Written informed consent was obtained from all participants. All relatives from 77 multiplex families were typed for 26 mapped microsatellite markers with an average resolution of 1 cM between SPN and D16S408. We constructed contigs using seven previously localized sequence tag sites (STSs; D16S541, D16S3085, D16S3136, D16S3117, D16S770, D16S416, D16S2623) and subsequently eight additional ones (wi-9288, wi-16305, shgc-17274, shgc-31023, shgc-32374, stSG-30035, wi-5812, D16S766) and 79 new STSs derived from the end sequences of the isolated BAC clones[6].

Clones, sequencing and SNPs

The DNA of BAC clone hb87b10 containing D16S3136 was fragmented by sonication and subcloned in bacteriophage M13. We used sequences from both strands of 706 subclones and from direct primer walking to reconstruct the initial BAC sequence using PhredPhrap (http://www.phrap.org). Identity search in DNA databases identified two overlapping sequenced BACs (AC007334, GenBank; AC007728, GenBank). Homology search, performed on the extended sequence with BLAST v1.4 in GenBank release 114, identified 10 Unigene clusters. The following EST clones corresponding to some of these clusters were obtained from the American Type Culture Collection (http://www.atcc.org) and sequenced completely to identify additional transcribed regions: AI125217, AA417810, AI375427, AA021341, AI090427, AA910520, AA731089. Clones AI090427 and AA910520, corresponding to hs135201, were used to screen a blood leukocyte cDNA library (no. 938202; Stratagene), and retrieved 11 clones of the *IBD1* candidate gene.

A total of 123 STSs, mostly selected from putatively transcribed sequences (EST homologies and GRAIL v2.0 predicted exons), including 11 exons of KIAA0849, was

sequenced following amplification from the DNA of ten CD patients and two unaffected individuals. Of 55 identified SNPs, SNP 1–11, selected for their rare-allele frequencies greater than 0.06, were typed on 1,272 members of the 235 CD families. SNP 12 and 13 were further identified by sequencing the 11 exons of the candidate *IBD1* gene in 50 CD patients (SNP 1–13, GenBank accession numbers G67943–G67955, are submitted to dbSNP of the National Center for Biotechnology Information) and typed on the same group of individuals. To search for rare variant alleles, we subsequently investigated the 11 exons of 457 CD patients, 159 ulcerative colitis patients and 103 unaffected unrelated individuals. All variant alleles were confirmed by sequencing a second independent amplification product.

Data analysis

Genotypic data were analysed for linkage using the NPL score of GeneHunter v2.0. Data from linkage disequilibrium mapping of CD were analysed initially with the transmission disequilibrium test[7] using a single trio (one affected and both parents) per family. Subsequently, the pedigree disequilibrium test was performed using the PDT 2.11 program[8] to analyse data from all family relatives. We estimated allele frequencies for 3 groups, 418 unrelated CD patients, 159 ulcerative colitis patients and 103 controls (including 78 unaffected, unrelated spouses of CD patients and 25 unrelated CEPH family members).

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A frameshift mutation in *NOD2* associated with susceptibility to Crohn's disease

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Crohn's disease is a chronic inflammatory disorder of the gastrointestinal tract, which is thought to result from the effect of environmental factors in a genetically predisposed host. A gene location in the pericentromeric region of chromosome 16, *IBD1*, that contributes to susceptibility to Crohn's disease has been established through multiple linkage studies[1–6], but the specific gene(s) has not been identified. *NOD2*, a gene that encodes a protein with homology to plant disease resistance gene products is located in the peak region of linkage on chromosome 16 (ref. 7). Here we show, by using the transmission disequilibrium test and case-control analysis, that a frameshift mutation caused by a cytosine insertion, 3020insC, which is expected to encode a truncated NOD2 protein, is associated with Crohn's disease. Wild-type NOD2 activates nuclear factor NF-κB, making it responsive to bacterial lipopolysaccharides; however, this induction was deficient in mutant NOD2. These results implicate *NOD2* in susceptibility to Crohn's disease, and suggest a link between an

sequenced following amplification from the DNA of ten CD patients and two unaffected individuals. Of 35 identified SNPs, SNP1–13 selected for their rare-allele frequencies greater than 0.05 were typed on 1272 members of the 235 CD families. SNP 12 and 13 were further identified by sequencing the 11 exons of the candidate *IBD1* gene in 50 CD patients (SNP 1–13, GenBank accession numbers G67949–G67955, are submitted to dbSNP of the National Center for Biotechnology Information) and typed on the same group of individuals. To search for rare variant alleles, we subsequently investigated the 11 exons of 457 CD patients, 159 ulcerative colitis patients and 103 unaffected unrelated individuals. All variant alleles were confirmed by sequencing a second independent amplification product.

Data analysis

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A frameshift mutation in *NOD2* associated with susceptibility to Crohn's disease

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Crohn's disease is a chronic inflammatory disorder of the gastrointestinal tract, which is thought to result from the effect of environmental factors in a genetically predisposed host. A gene location in the pericentromeric region of chromosome 16, *IBD1*, that contributes to susceptibility to Crohn's disease has been established through multiple linkage studies[1–6], but the specific gene(s) has not been identified. *NOD2*, a gene that encodes a protein with homology to plant disease resistance gene products is located in the peak region of linkage on chromosome 16 (ref. 7). Here we show, by using the transmission disequilibrium test and case-control analysis, that a frameshift mutation caused by a cytosine insertion, 3020insC, which is expected to encode a truncated NOD2 protein, is associated with Crohn's disease. Wild-type NOD2 activates nuclear factor NF-κB, making it responsive to bacterial lipopolysaccharides; however, this induction was deficient in mutant NOD2. These results implicate *NOD2* in susceptibility to Crohn's disease, and suggest a link between an

innate immune response to bacterial components and development of disease.

The idiopathic inflammatory bowel diseases (IBDs), which include Crohn's disease (CD) and ulcerative colitis, are chronic disorders of the gastrointestinal tract with unknown aetiology, and with a combined prevalence of about 150–200 cases per 100,000 in western countries[8,9]. Although the aetiology of IBD is unknown, an abnormal inflammatory response directed against enteric microflora in a genetically susceptible host has been proposed[10]. Familial clustering of disease and studies of twins strongly suggest that IBD, and in particular CD, is a genetic disorder[11]. Genome-wide searches for IBD-susceptibility genes have resulted in the identification of several loci for CD and/or ulcerative colitis, most notably for CD, in the pericentromeric region of chromosome 16 (*IBD1*)[1–6].

NOD2 has structural homology with both the apoptosis regulators Apaf-1/Ced-4 and a class of plant disease resistant (R) gene products[7]. Like the latter gene products, NOD2 comprises an amino-terminal effector domain, a nucleotide-binding domain and leucine-rich repeats (LRRs) (ref. 7). *NOD2* has been mapped to chromosome 16q12 (ref. 7) and is tightly linked to markers D16S3396, D16S416 and D16S419 (Fig. 1a)—a site that precisely overlaps with *IBD1* (ref. 1). Given the genomic localization and the role of NOD proteins in recognizing bacterial components[12], we thought that *NOD2* might function as a susceptibility gene for CD.

The 12-exon genomic organization of the *NOD2* gene was determined by aligning the complementary DNA sequence (AF178930) with one genomic bacterial artificial chromosome (BAC) clone, RP11-327F22 (AC007728) (Fig. 1a). All coding exons and flanking introns were sequenced in 12 affected individuals from pure CD families with increased linkage scores at D16S3396, as well as in 4 case controls. In three CD patients, a cytosine insertion was observed in exon 11 at nucleotide 3020 (3020insC) (Fig. 1b). 3020insC resulted in a frameshift at the second nucleotide of codon 1007 (Fig. 1b), and a Leu1007→Pro substitution in the tenth LRR, followed by a premature stop codon (Fig. 1c). The predicted truncated NOD2 protein contained 1,007 amino acids instead of the 1,040 amino acids of the wild-type NOD2 protein (Fig. 1d).

We used an allele-specific polymerase chain reaction (PCR) assay (Fig. 2) to type 3020insC in IBD families and case controls. Analysing only one CD patient per independent family, we observed preferential transmission of 3020insC (39 transmissions and 17 non-transmissions; $P = 0.0046$). Analysing all independent nuclear families by sib-TDT (ftp://lahmed.stanford.edu/pub/aspex/index.html), the empirical P value was similar, $P = 0.0007$. As expected from linkage studies[2,5], no preferential transmission of 3020insC was observed among families with ulcerative colitis (data not shown). As 365 of the 416 independent CD families have several affected individuals, the applicability of these associations to the more common, sporadic cases requires further study.

Additional support for association to CD was provided by case-

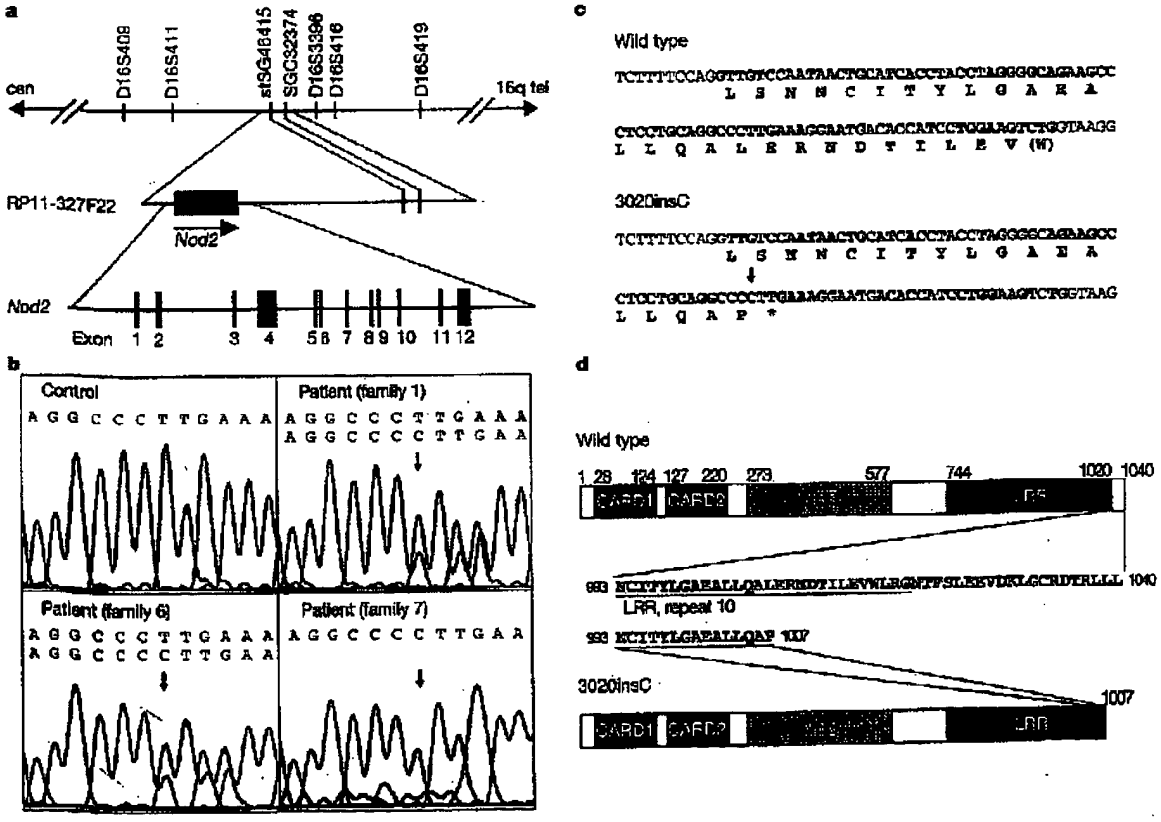

Figure 1 Identification of a frameshift *NOD2* mutation in affected individuals from CD families. **a**, Physical map of the region of interest at 16q12. Approximate positions of chromosomal and genetic markers are based on ref. 22. The human genomic BAC clone RP11-32722 contains the *NOD2* gene and markers stSG46415 and SGC32374. The exon–intron organization of the human *NOD2* gene is shown underneath. **b**, DNA sequence electropherograms (exon 11) from control and three affected individuals from [illegible]. Patients from families 1 and 6 are heterozygous, whereas the patient from [illegible] homozygous for 3020insC. The cytosine insertion is indicated by an arrow. **c**, Nucleotide and predicted amino-acid sequence of exon 11 and flanking introns from wild-type control and patients with 3020Cins. The exon sequence is shown in bold. The site of 3020insC is indicated by an arrow. Residue (W) indicates that a nucleotide from exon 12 contributes to the codon. **d**, Domain structure of NOD2, illustrating the site of protein truncation. Caspase-recruitment domains (CARDs), the nucleotide-binding domain (NBD) and ten LRRs are shown. Residues of the tenth LRR are underlined. Numbers indicate residue positions.

Table 1 TDT demonstrates preferential transmission of the 3020insC to CD patients

	One CD patient per family			All CD patients	
Source	Transmitted	Not transmitted	P value	Transmitted	Not transmitted
Univ. of Chicago	21	10		32	16
Johns Hopkins	4	4		10	8
Univ. of Pittsburgh	14	3		26	9
Total	39	17	0.0046	68	33

Table 2 Allele frequency of 3020insC in unrelated Crohn's disease patients and controls

	Crohn's disease			Case controls	
Source	Sample size	3020insC*	Source	Sample size	3020insC*
Univ. of Chicago	212	7.3	Chicago	65	3.8
Johns Hopkins	88	6.8	Baltimore	46	3.2
Univ. of Pittsburgh	118	10.8	San Francisco	81	3.1
			Germany	94	5.3
Total	418	8.2		287	4.0

For analysis, one CD patient was selected from 418 independent families. the difference in allelic frequency between CD patient and control was significant ($P = 0.0018$, by the large sample approximations to a two sample binomial test).
*Per cent allele frequency.

control analysis, in which, using one CD individual per independent family, the 3020insC allele frequency among all CD groups was 8.2% (Table 2). The allele frequencies of 3020insC were comparable among Jewish (8.4%) and non-Jewish Caucasians (8.1%). Among case controls (Table 2), the allele frequency in four separate Caucasian cohorts of 4.0% was significantly lower than in CD patients ($P = 0.0018$, by large-sample approximations to a two-sample binomial test). The allele frequency of the 3020insC among 182 unrelated ulcerative colitis patients was 3.0%, and was significantly lower than the frequency among CD patients ($P = 0.0010$). The genotype frequencies of 3020insC in unrelated CD individuals were 11 homozygotes, 46 heterozygotes and 359 wild-type homozygotes.

Among case controls, there were 23 heterozygous individuals, with the remaining being wild-type homozygotes. The genotype-relative risk (GRR) for heterozygous and homozygous 3020insC was 1.5 and 17.6, respectively, as compared with wild-type controls. Given its frequency, 3020insC is unlikely to account completely for the observed evidence of linkage at *IBD1*, and other variants of *NOD2* may confer additional disease risk. For example, two single-nucleotide polymorphisms in *NOD2* have been identified, 2722G→C (Gly908Arg) and 2104C→T (Arg702Trp), which are highly associated with CD by the transmission disequilibrium test (data not shown). Furthermore, other susceptibility genes might also be present in this broad region[1–5] of linkage on chromosome 16.

NOD2 has been shown to activate NF-κB and to confer responsiveness to bacterial lipopolysaccharides[7,12]. To test the ability of wild-type and mutant NOD2 to activate NF-κB, human embryonic kidney (HEK) 293T cells were transiently co-transfected with wild-type or 3020insC plasmids and an NF-κB reporter construct. In the absence of lipopolysaccharide (LPS), expression of both wild-type and mutant NOD2 induced activation of NF-κB (Fig. 3a). Notably, equivalent levels of wild-type and mutant NOD2 protein expression (as assessed by immunoblotting of total lysates) resulted in similar levels of NF-κB activation (Fig. 3a).

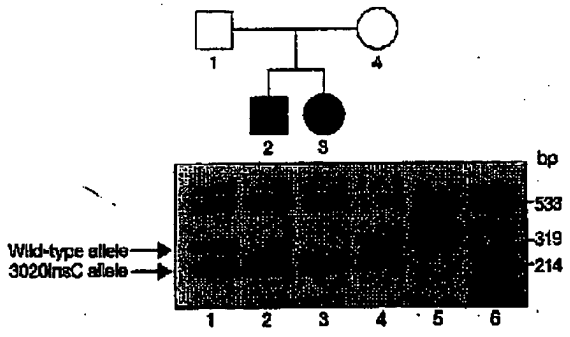

Figure 2 Determination of transmission of the 3020insC mutation in a CD family by allele-specific PCR. Multiplex PCR was used to generate a nonspecific 533-bp product, along with allele-specific amplicons: a 319-bp fragment (wild type) and a 214-bp fragment (3020insC). In this family, both parents (lanes 1 and 4) are heterozygous for 3020insC, whereas both children (lanes 2 and 3) have CD and are homozygous for 3020insC. Lane 5, wild-type control; lane 6, pBR322 DNA MspI markers. Numbers on the right indicate the size of fragments.

Like NOD2, cytosolic plant disease resistant proteins have carboxy-terminal LRRs that are critical for the recognition of pathogen components and induction of pathogen-specific responses[13–15]. We therefore compared the ability of wild-type and mutant NOD2 proteins to induce NF-κB activity in response to LPS. Because overexpression of NOD2 induces potent NF-κB activation (Fig. 3a), we transfected the cells with low amounts of wild-type and mutant

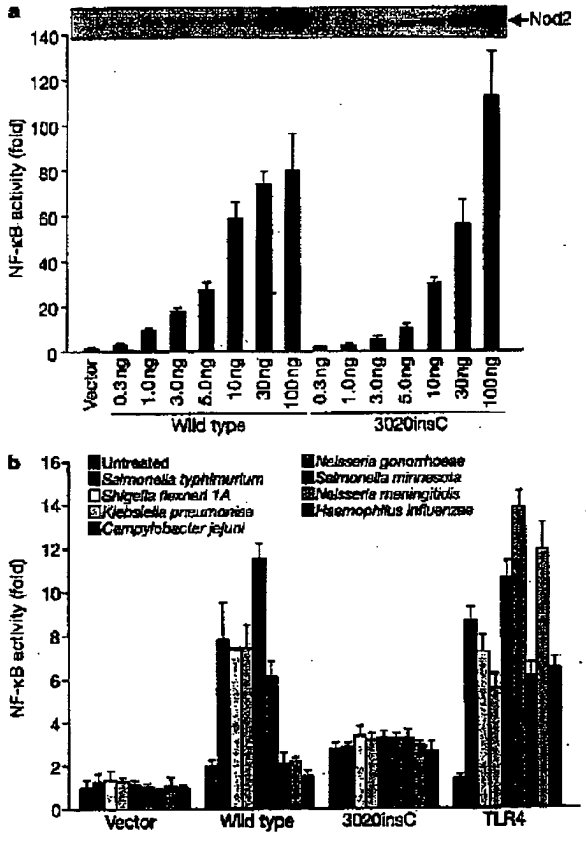

Figure 3 Differential responsiveness of wild-type and mutant NOD2 to LPS. a, HEK293T cells were co-transfected in triplicate with the indicated amounts of pcDNA3 (vector), wild-type pcDNA3-NOD2, or pcDNA3-NOD2 3020insC and pEF-BOS-β-gal and pBVI-luc reporter plasmids. Values represent means ± s.d. Expression of wild-type and mutant NOD2 proteins in cell extracts is shown on top. b, HEK293T cells were co-transfected in triplicate with 0.3 ng of pcDNA3-NOD2, 3 ng of pcDNA3-NOD2 3020insC, 3 ng of pcDNA3-TLR4 plus 3 ng of pcDNA3-MD-2 (indicated by TLR4) or pcDNA3 (vector) and pEF-BOS-β-gal and pBXIV-luc. Under these conditions, both wild-type and mutant NOD2 constructs induced similar levels of basal NF-κB activity. Eight hours after transfection, cells were treated with 10 μg ml^{-1} of LPS from indicated bacteria. Values represent means ± s.d. Results are representative of at least five independent experiments.

NOD2 plasmids to induce similar levels of protein expression and basal NF-κB activity (Fig. 3a). LPS from various bacteria induced NF-κB activation in cells expressing wild-type NOD2, but not in cells transfected with control plasmid (Fig. 3b).

Significantly, the ability of mutant NOD2 to confer responsiveness to LPS was greatly diminished when compared with wild-type NOD2 (Fig. 3b). Differential regulation of NOD2 function by LPS from different bacteria was observed (Fig. 3b), whereas all LPS preparations induced NF-κB activation comparably in cells transfected with Toll-like receptor-4 (TLR-4), a cell-surface receptor for LPS[16].

The innate immune system regulates the immediate response to microbial pathogens and is initiated by recognition of specific pathogen components by receptors in host immune cells[16]. NOD1 and NOD2 seem to function as intracellular receptors for LPS with the LRRs required for responsiveness[12]. We have shown here that truncation of the tenth LRR of NOD2 is associated with CD. Consistent with earlier linkage studies[2,5], this variant is associated solely with CD, and not with ulcerative colitis. Functional analyses indicate that the disease-associated NOD2 variant is significantly less active than the wild-type protein in conferring responsiveness to bacterial LPS. In plant NOD2 homologues, the LRRs determine the specificity for pathogen products and alterations in LRRs can result in unresponsiveness to particular pathogens[13–15]. Similarly, genetic variation in the LRRs of TLR4 account for inter-individual differences in bronchial responsiveness to aerosolized LPS[17].

Several mechanisms can be envisaged to account for susceptibility to CD in individuals carrying this variant. NOD2 is a cytosolic protein whose expression is restricted to monocytes, with no expression detected in lymphocytes[7]. A deficit in sensing bacteria in monocytes/macrophages might result in an exaggerated inflammatory response by the adaptive immune system. A related possibility is that wild-type NOD2 may mediate the induction of cytokines such as interleukin-10 that can downregulate the inflammatory response[18,19]. Finally, variation in the LRRs of plant NOD2 homologues can result in recognition of new specificities for pathogen components[13,14]. Thus, it is also possible that NOD2 variants might act as gain-of-function alleles for unknown pathogens. Our studies implicate *NOD2* in susceptibility to CD, and suggest a link between an innate response to bacterial components and development of disease. □

Methods

IBD families

IBD families were ascertained for linkage and association studies (affected child with both parents) through the University of Chicago, the Johns Hopkins Hospital and the University of Pittsburgh. In all cases informed consent for a molecular genetic study was obtained, and the study protocol was approved by the individual institutional review boards.

Allele-specific PCR

We used primers framing a 539-base-pair region surrounding the 3020insC allele to amplify genomic DNA isolated from controls and patients by PCR (sense, 5′-CTGAGCCTTTGTTGATGAGC-3′; antisense, 5′-TCTTCAACCACATCCCCATT-3′). In addition, each PCR reaction contained two additional primers designed to detect the wild-type allele (sense, 5′-CAGAAGCCCTCCTGCAGGCCT-3′) and another primer designed to detect the 3020insC allele (antisense, 5′-CGCGTGTCATTCCTTTCAT GGGGC-3′). The 3020insC was confirmed by DNA sequencing. We performed multiplex PCR with all four primers in one tube. PCR products were isolated on 2% agarose gels and visualized with ethidium bromide.

Data analysis

The *P* values for the TDT test[20] were calculated using a binomial exact test. Simulations (500,000 replicates) were done using the sib-TDT software (ftp://lahmed.stanford.edu/pub/asper/index.html) to calculate empirical probabilities for the TDT χ^2 statistic when all independent nuclear families were counted. This calculation was done by permuting parent alleles while fixing the IBD status of siblings within a family. We estimated the frequency of the genotypes in the affected individuals from 416 unrelated CD patients. The GRR was defined as the ratio of the marginal penetrance of the 3020insC homozygote [illegible] to the wild-type homozygotes. Using Bayes rule, the GRRs can [illegible] control groups. For the control group, we assumed that the alleles are in Hardy–Weinberg equilibrium.

Expression plasmids and immunoblotting

The expression plasmids pcDNA3-NOD2, pcDNA3-TLR4 and pcDNA3-MD-2 have been described[7,12]. The expression plasmid producing the NOD2ΔL33 mutant (3020insC) was generated by PCR and cloned into pcDNA3 (Invitrogen), and confirmed by DNA sequencing. Expression of untagged NOD2 proteins in transfected cells was determined by immunoblotting using affinity-purified rabbit anti-NOD2 antibody, as described[7]. To raise the antibody, we overexpressed recombinant NOD2 protein (residues 28–301) in *Escherichia coli* strain BL21(DE3) using the pET-30a vector (Novagen). Recombinant NOD2 protein containing a C-terminal histidine tag was purified using a nickel column (Novagen) and injected into rabbits.

NF-κB activation assay

We carried out NF-κB activation assays as described[7,12]. Briefly, HEK293T cells were co-transfected with 12 ng of the reporter construct pBVI-Luc, the indicated amounts of each expression plasmid and 120 ng of pEF-BOS-β-gal in triplicate in the presence or absence of LPS[7,12]. LPS from various sources were obtained from Sigma or from several investigators. Twenty-four hours after transfection, cell extracts were prepared and the relative luciferase activity was measured as described[7,12]. Results were normalized for transfection efficiency with values obtained with pEF-BOS-β-gal.

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Exhibit D

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Genetic approaches in mice to understand Rel/NF-κB and IκB function: transgenics and knockouts

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Rel/NF-κB transcription factors have been implicated in regulating a wide variety of genes important in cellular processes that include cell division, cell survival, differentiation and immunity. Here genetic models in which various Rel/NF-κB and IκB proteins have either been over-expressed or deleted in mice will be reviewed. Although expressed fairly ubiquitously, homozygous disruption of individual Rel/NF-κB genes generally affects the development of proper immune cell function. One exception is *rela*, which is essential for embryonic liver development. The disruption of genes encoding the individual subunits of the IκB kinase, namely IKKα and IKKβ, has demonstrated that IKKβ transmits the response to most common NF-κB inducing agents, whereas IKKα has an unexpected role in keratinocyte differentiation. Future studies will no doubt focus on the effect of multiple gene disruptions of members of this signaling pathway, on tissue-specific disruptions of these genes, and on the use of these mice as models for human diseases.

Keywords: NF-κB; Rel; IκB; IKK; knockout mice; transgenic mice; mouse genetics

Introduction

In mammals, there are five distinct Rel/NF-κB transcription factor subunits—p50/p105, p52/p100, c-Rel, RelA and RelB—each encoded by a unique gene. Two genes, *nfkb1* and *nfkb2*, encode large cytoplasmic proteins (p105 and p100, respectively) with inhibitor IκB properties, and smaller DNA-binding subunits (p50 and p52, respectively) that correspond to the conserved N-terminal domain shared by all Rel/NF-κB proteins. The proteins encoded by c-*rel*, *rela* and *relb* contain C-terminal transcriptional transactivation domains in addition to their Rel Homology (RH) domains. Rel/NF-κB proteins bind to specific DNA target sites (κB sites) as heterodimers or homodimers. The most common complex in many cells is the p50-RelA heterodimer, usually specifically referred to as NF-κB. Rel/NF-κB dimers usually do not promote transcription if they lack a subunit with a C-terminal transactivation domain.

In most cell types, Rel/NF-κB transcription complexes are present as latent, cytoplasmic forms, which can be induced to enter the nucleus and activate gene expression. The cytoplasmic sequestration of Rel/NF-κB is regulated by the family of IκB inhibitor proteins that includes IκBα, IκBβ, IκBγ, IκBε and Bcl-3. Two major kinases, IKKα and IKKβ are responsible for the induced phosphorylation of IκBα and IκBβ, and this phosphorylation targets for IκBs for proteasome-dependent degradation and thus releases the Rel/NF-κB complex. The generation of transgenic mice over-expressing exogenous copies of particular Rel/NF-κB or IκB genes, and of mice homozygous for null mutations in Rel/NF-κB, IκB or IKK genes, has provided invaluable models for elucidating the physiological functions and regulation of the components in the Rel/NF-κB signal transduction pathway. The phenotypes of the various transgenic and mutant mouse models are summarized herein.

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Null mutations for Rel/NF-κB proteins

Single mutations

nfkb1$^{-/-}$ *mice* *nfkb1* encodes primarily two proteins, a 105 kDa non-DNA binding cytoplasmic molecule (p105) and a 50 kDa DNA-binding protein (p50) that corresponds to the N terminus of p105. In addition, in certain mouse cells, an *nfkb1*-encoded protein (IκBγ) containing only the C-terminal half of p105 has been detected (Inoue *et al.*, 1992). Despite the nearly ubiquitous expression of *nfkb1* and the role of p50 as the major partner of RelA, which is required for normal embryogenesis (see below), mice lacking p50 and p105 (*nfkb1*$^{-/-}$ mice) develop normally and exhibit no histopathological changes. Although p50/p105 is not essential for hemopoiesis, *nfkb1*$^{-/-}$ mice exhibit multiple defects in the function of the immune system (Sha *et al.*, 1995). Mature quiescent *nfkb1*$^{-/-}$ B cells turn over more rapidly *in vivo* and undergo accelerated apoptosis in culture (Grumont *et al.*, 1998), indicating that p50/p105 is required for the survival of non-activated B cells. When activated with lipopolysaccharide and soluble CD40 ligand, *nfkb1*$^{-/-}$ splenic B cells proliferate poorly. However, these cells respond normally to antigen receptor ligation and anti-IgD dextran antibodies (Sha *et al.*, 1995; Snapper *et al.*, 1996), indicating that p50/p105 is only essential for certain B-cell activation pathways. The B-cell proliferative defects in *nfkb1*$^{-/-}$ mice are due to a cell-cycle block in G1 and enhanced mitogen-induced apoptosis (Grumont *et al.*, 1998).

Consistent with a role for *nfkb1* in B-cell activation, *nfkb1*$^{-/-}$ mice fail to mount a normal humoral

response when challenged with various antigens (Sha *et al*., 1995). This is due, in part, to cell autonomous defects in heavy chain isotype switching resulting from impaired transcriptional induction of germline C_H genes by mitogens and cytokines (Snapper *et al*., 1996), the expression of which is required for immunoglobulin gene rearrangement. Interestingly, the susceptibility of *nfkb1*$^{-/-}$ mice to various pathogens varies quite markedly. These mice are more susceptible to *Listeria monocytogenes* and *Streptococcus pneumoniae*, respond normally to *Haemophilus influenza* and *Escherichia coli*, and are more resistant to murine encephalomyocarditis (EMC) virus (Sha *et al*., 1995). Enhanced resistance to EMC correlates with increased production of β-interferon by *nfkb1*$^{-/-}$ cells (Sha *et al*., 1995), thereby implicating p50/p105 as a regulator of innate as well adaptive immune responses.

nfkb2$^{-/-}$ *mice* Similar to *nfkb1*, the *nfkb2* gene encodes a 52 kDa protein that corresponds to the N-terminus of a larger 100 kDa protein. However, unlike *nfkb1*, *nfkb2* expression is restricted to the epithelium of the stomach and select areas of hemopoietic organs such as the thymic medulla, the marginal zone and periarterial sheath of the spleen (Attar *et al*., 1997). Mice lacking p52/p100 proteins develop normally, with the major defect being a disruption of splenic and lymph node architecture (Caamaño *et al*., 1998; Franzoso *et al*., 1998). In the spleen of these mice, the perifollicular marginal zone, thought to be important for regulating cell migration during immune responses, is absent and the B-cell follicular areas are either absent or depleted. Although the failure of these mice to mount a normal T cell-dependent antibody response is associated with an inability to form germinal centers, this cannot simply be explained by intrinsic B- or T-cell defects, as *nfkb2*$^{-/-}$ lymphocytes exhibit only mildly impaired proliferative responses coupled with normal antibody or cytokine production when activation in culture (Franzoso *et al*., 1998). Instead, these immune deficiencies appear to reflect a defect in antigen presentation by accessory cells. A role for p52/p100 in the regulation of antigen presentation is consistent with the findings that the hemopoietic restricted sites of *nfkb2* expression contain macrophages and dendritic cells and that p52 is a major dimer partner of RelB, another Rel/NF-κB family member important for the function and development of dendritic cells.

c-rel$^{-/-}$ *mice* Restricted expression of c-Rel to lymphocytes, monocytic, granulocytic, and erythroid cells in mouse fetal and adult hemopoietic organs coincides with the development and expansion of the hemopoietic system. Consistent with this pattern of expression, c-Rel is essential for a variety of functions in hemopoietic cells, although it is dispensable for mouse embryonic development. Although normal numbers of hemopoietic cells in c-*rel*$^{-/-}$ mice indicate that c-Rel is not essential for the differentiation of hemopoietic precursors, mature lymphocytes and macrophages exhibit a number of activation-associated defects associated with B- and T-cell proliferation, isotype switching and the production of various cytokines and immune modulatory molecules (Gerondakis *et al*., 1996; Grigoriadis *et al*., 1996; Grumont *et al*., 1998; Köntgen *et al*., 1995).

Impaired c-*rel*$^{-/-}$ B-cell proliferation in response to range of individual mitogens is due to a cell-cycle block in G1 and elevated activation-induced apoptosis (Grumont *et al*., 1998). While c-Rel-regulated gene(s) critical for cell-cycle progression remained to be determined, mitogen-induced apoptosis in c-*rel*$^{-/-}$ B cells is due in part to a failure to upregulate the expression of *A1*, a Bcl-2 prosurvival homologue directly regulated by c-Rel (Grumont *et al*., 1999). In c-*rel*$^{-/-}$ B cells, defects in immunoglobulin C_H switching implicate c-Rel in various steps of the switching process such as germline C_H gene transcription. In contrast to the cell autonomous defects that afflict c-*rel*$^{-/-}$ B-cell proliferation, the failure of c-*rel*$^{-/-}$ T cells to proliferate in response to mitogens can be overcome by exogenous interleukin-2 (IL-2) (Gerondakis *et al*., 1998). IL-2 is not, however, the only cytokine whose expression is impaired, as activated c-*rel*$^{-/-}$ T cells also express reduced amounts of IL-3 and GM–CSF (Gerondakis *et al*., 1996). c-Rel also serves distinct roles in different macrophage populations. Whereas GM-CSF, G-CSF, IL-6, TNFα and iNOS expression is abnormal in activated c-*rel*$^{-/-}$ resident peritoneal macrophages, only TNFα and IL-6 expression is impaired in stimulated c-*rel*$^{-/-}$ elicited peritoneal macrophages (Grigoriadis *et al*., 1996). An elevation of GM-CSF expression in certain c-*rel*$^{-/-}$ macrophage populations (Grigoriadis *et al*., 1996), but reduced GM–CSF expression in c-*rel*$^{-/-}$ T cells (Gerondakis *et al*., 1996) highlights the tissue-specific modulation of c-Rel function and establishes that mammalian c-Rel, like *Drosophila* Dorsal (see Govind, 1999), is both an activator and repressor of gene expression. *In vitro* defects in c-*rel*$^{-/-}$ lymphocyte and macrophate function are also reflected in the impaired innate and adaptive immune response of c-Rel-deficient mice (Gerondakis *et al*., 1996; Harling-McNabb *et al*., 1999; Köntgen *et al*., 1995).

rela$^{-/-}$ *mice* The absence of RelA leads to embryonic lethality between days E15 and E16 post-coitum, a result of fetal hepatocyte apoptosis (Beg and Baltimore, 1996). The death of *rela*$^{-/-}$ fetal hepatocytes arises from their heightened sensitivity to the cytotoxic effects of TNFα, as evidenced by the observation that an absence of this cytokine rescues *rela*$^{-/-}$ mice from embryonic lethality (Doi *et al*., 1999). Consistent with RelA playing an anti-apoptotic role in different cell types, *rela*$^{-/-}$ fibroblasts and macrophages also exhibit increased sensitivity to apoptosis induced by TNFα (Beg and Baltimore, 1996). Although several Rel/NF-κB regulated prosurvival genes including *A1* (Zong *et al*., 1999), *cIAP2* (Wang *et al*., 1998) and *IEX-1L* (Wu *et al*., 1998) are normally upregulated by TNFα, it remains to be determined which, if any, of these is critical in protecting hepatocytes from TNFα-induced apoptosis (see also Barkett and Gilmore, 1999). RelA is also important for normal lymphocyte function. The analysis of SCID mice reconstituted with day E13 *rela*$^{-/-}$ fetal liver cells has established that RelA, while dispensable for lymphopoiesis, is required for mitogen-induced lymphocyte proliferation and isotype switching (Doi *et al*., 1997).

relb$^{-/-}$ *mice* RelB expression is normally confined to dendritic cells and B lymphocytes. Mice lacking RelB

exhibit multiple pathological lesions and defects in acquired and innate immunity. Pathological conditions that afflict *relb*$^{-/-}$ mice include T-cell inflammatory infiltrates of various organs, T cell-dependent myeloid hyperplasia and splenomegaly due to extramedullary hemopoiesis (Burkly *et al.*, 1995; Weih *et al.*, 1995). The basis of the inflammatory pathology in *relb*$^{-/-}$ mice remains unclear, but may be due to the absence of certain thymic and splenic dendritic cell (DC) populations (Wu *et al.*, 1998). Although the thymic DC deficiency in *relb*$^{-/-}$ mice is a secondary consequence of the disrupted thymic architecture, the absence of splenic myeloid related CD8α DCs is a direct stem cell intrinsic defect (Wu *et al.*, 1998). This deficit of thymic and splenic DCs may account for the inability of *relb*$^{-/-}$ mice to effectively delete autoreactive thymocytes and T cells (Burkly *et al.*, 1995), which in turn creates a pool of self-reactive T cells that ultimately give rise to the inflammatory phenotype. Delayed-type hypersensitivity and CD8$^+$ cytotoxicity, macrophage-mediated immunity to various pathogens, and IgG responses to T cell-dependent antigens are also impaired (Weih *et al.*, 1997; Caamaño *et al.*, 1999). Because delayed-type hypersensitivity responses are dependent on Langerhans cells, this may indicate that these antigen-presenting cells are also defective in *relb*$^{-/-}$ mice. The findings that the proliferative response of *relb*$^{-/-}$ splenic B cells to mitogens in tissue culture is only reduced slightly and that Ig secretion and isotype switching are normal indicate that the humoral immune defects are most likely a secondary consequence of impaired CD4$^+$ T-cell function arising from deficiencies in antigen presentation. These findings indicate that in contrast to the other Rel/NF-κB proteins, each of which is dispensable for normal hemopoiesis, RelB is required for the development of specific dendritic cell populations.

Multiple mutations

Despite the unique roles ascribed to individual Rel/NF-κB subunits, functional overlap and redundancy among these proteins most likely prevents the emergence of certain phenotypes in single mutant mice due to compensation by other family members. Indeed, mice lacking multiple Rel/NF-κB proteins often exhibit novel phenotypes or more severe versions of those phenotypes seen in single mutants.

nfbk1$^{-/-}$ nfkb2$^{-/-}$ *mice* Mice deficient in both *nfkb1* and *nfkb2*, while phenotypically indistinguishable from control litter mates at birth, soon exhibit growth retardation and cranofacial abnormalities, the latter being a result of bone thickening due to osteopetrosis (Franzoso *et al.*, 1997; Iotsova *et al.*, 1997). Bone remodeling is dependent on bone resorption by myeloid lineage-derived osteoclasts. Although osteoclast numbers are markedly reduced in these double mutant mice and *nfkb1*$^{-/-}$ *nfkb2*$^{-/-}$ osteoclast progenitors cannot differentiate *in vitro* (Iotsova *et al.*, 1997), transplantation of normal marrow into newborn *nfkb1*$^{-/-}$ *nfkb2*$^{-/-}$ mice only partially rescues this osteopetrotic phenotype (Franzoso *et al.*, 1997; Iotsova *et al.*, 1997). This indicates that the combined deficiency of *nfkb1* and *nfkb2* afflicts cells of a hemopoietic origin and the bone marrow microenvironment. Target gene(s) important for normal bone development that are affected by the absence of *nfkb1* and *nfkb2* remain to be identified.

In contrast to the single mutant mice, B-cell development is blocked in *nfkb1*$^{-/-}$ *nfkb2*$^{-/-}$ double-mutant mice at the immature IgM$^+$IgD$^-$ stage (Franzoso *et al.*, 1997), which normally corresponds to those cells newly emerged from the bone marrow. Collectively, these findings indicate that these two non-transactivating NF-κB proteins perform redundant functions in bone development and B-cell differentiation.

nfkb1$^{-/-}$ relb$^{-/-}$ *mice* The absence of *nfkb1*-encoded proteins exacerbates the severity and extent of organ inflammation resulting from an absence of RelB, with the mice dying within 3–4 weeks of birth (Weih *et al.*, 1997). While myeloid hyperplasia is more pronounced, the inflammatory infiltrates in *nfkb1*$^{-/-}$ *relb*$^{-/-}$ mice are devoid of B cells, the result of a B-cell developmental defect that leads to a marked reduction in both B220$^+$ splenic and bone marrow cells. This phenotype indicates that p50-containing complexes partly compensate for RelB function in dendritic cells and that p50/p105 and RelB perform redundant functions in B-cell development.

nfkb1$^{-/-}$ *c*-rel$^{-/-}$ *mice* Embryogenesis is normal in mice that lack p50 and c-Rel (Pohl *et al.*, in preparation). While both transcription factors are also dispensable for the differentiation of hemopoietic precursors, immune defects in these double mutants are more severe than in individual c-*rel*$^{-/-}$ or *nfkb1*$^{-/-}$ mice (Pohl *et al.*, in preparation). *nfkb1*$^{-/-}$ c-*rel*$^{-/-}$ lymphocytes fail to divide when stimulated with any combination of mitogens. Whereas *nfkb1*$^{-/-}$ c-*rel*$^{-/-}$ B cells cannot exit the G0 stage of the cell cycle when treated with mitogens, *nfkb1*$^{-/-}$ c-*rel*$^{-/-}$ T cells can undergo blast formation, indicating that the G0-G1 transition is differentially regulated by Rel/NF-κB in B and T cells. Consistent with the increased severity of the *nfkb1*$^{-/-}$ c-*rel*$^{-/-}$ lymphocyte activation defects, humoral immunity is further diminished in the double-mutant mice. This is due in part to the lack of germinal centers and an absence of germline C_H gene expression. This demonstrates that functional redundancy of c-Rel and p50 dimers is only important in the immune system.

rela$^{-/-}$ *c*-rel$^{-/-}$ *mice* *rela*$^{-/-}$ c-*rel*$^{-/-}$ double mutants, like *rela*$^{-/-}$ single mutant mice, die as a result of fetal hepatocyte apoptosis, with the onset of liver degeneration occurring 1–1.5 days earlier in gestation (E13–E13.5) than in *rela*$^{-/-}$ single mutants (Grossmann *et al.*, 1999) This indicates that c-Rel partly compensates for the anti-apoptotic function of RelA, which is consistent with the anti-apoptotic effects of c-Rel and RelA *in vitro* (see Barkett and Gilmore, 1999). The combined loss of c-Rel and RelA is also associated with multiple hemopoietic cell defects (Grossmann *et al.*, 1999). Lethally-irradiated mice engrafted with E12 *rela*$^{-/-}$ c-*rel*$^{-/-}$ fetal liver hemopoietic precursors die from the combined effects of anemia and granulocytosis. The anemia in reconstituted mice appears to reflect a defect in erythrocyte differentiation rather than a reduction in erythroid progenitors, as the

number of erythroid colonies in cultures of double mutant fetal liver cells is normal. Consistent with a developmental defect in erythropoiesis, the fetal blood of E13 *rela*$^{-/-}$ c-*rel*$^{-/-}$ embryos has higher than normal numbers of nucleated embryonic erythrocytes. This persistence of embryonic erythrocytes in *rela*$^{-/-}$ c-*rel*$^{-/-}$ embryos suggests that the switch from primitive to definitive erythropoiesis is impaired.

Monocyte differentiation is also affected in these mice. The loss of both c-Rel and RelA results in cell death during monocyte differentiation in culture. In contrast, c-*rel*$^{-/-}$ *rela*$^{-/-}$ monocytic precursors appear to differentiate normally *in vivo*, indicating that compensatory signals or factors critical for monocyte differentiation and survival that are missing *in vitro* operate *in vivo*.

Although the combined loss of c-Rel and RelA does not impair thymocyte development or B-cell differentiation in the bone marrow, *rag1*$^{-/-}$ mice reconstituted with double mutant fetal liver hemopoietic precursors exhibit a marked reduction in the number of peripheral B and T cells (Grossmann *et al.*, in preparation). *rela*$^{-/-}$ c-*rel*$^{-/-}$ B cells (IgM$^+$IgD$^-$) newly emerged from the bone marrow fail to mature to IgM$^-$IgD$^+$ cells, a finding consistent with very low serum immunoglobulin levels and an absence of B cells in the lymph nodes of these mice (Grossmann *et al.*, 1999). The death of double mutant *rela*$^{-/-}$ c-*rel*$^{-/-}$ IgM$^+$IgD$^+$ B cells is dramatically accelerated *in vitro* and these cells turnover more rapidly than their normal or single mutant counterparts *in vivo*. Consequently, the failure of *rela*$^{-/-}$ c-*rel*$^{-/-}$ B cells to enter the mature B-cell pool after exiting the bone marrow appears to be due to a reduced lifespan. In contrast, the profound reduction of mature CD4$^+$ and CD8$^+$ T cells in the spleen and lymph nodes of engrafted *rag1*$^{-/-}$ mice is not associated with increased cell death; instead it appears to be linked to a defect in the post-thymic expansion of *rela*$^{-/-}$ c-*rel*$^{-/-}$ T cells. This indicates that although c-Rel and RelA are essential for the generation of mature B and T cells, these transcription factors only appear to be important for survival in B-cells

nfkb1$^{-/-}$ rela$^{-/-}$ *mice* The absence of both p50 and RelA (the NF-κB complex), like the combined loss of c-Rel and RelA, leads to an earlier onset of embryonic death (around E13) due to fetal liver apoptosis (Horvitz *et al.*, 1997). Irradiated mice engrafted with E12 *nfkb1*$^{-/-}$ *rela*$^{-/-}$ fetal liver hemopoietic precursors lack B220$^+$ cells in the bone marrow, spleen and blood, indicating that the defect in B lymphopoiesis occurred at a stage before the development of B220$^+$ B-cell precursors (Horvitz *et al.*, 1997). Simultaneous transplantation of wild-type bone marrow cells rescues the production of *nfkb1*$^{-/-}$ *rela*$^{-/-}$ B cells, a finding consistent with NF-κB mediating the development or survival of an early lymphocyte precursor by regulating an extracellular factor. B cells lacking both p50/p105 and RelA exhibit profound proliferative defects in response to mitogen stimulation (Horvitz *et al.*, 1999). Mice engrafted with NF-κB deficient fetal liver cells also develop a fetal liver-derived granulocytosis (Horvitz *et al.*, 1997). Combined with the observation that mice receiving *rela*$^{-/-}$ c-*rel*$^{-/-}$, but not *nfkb1*$^{-/-}$ c-*rel*$^{-/-}$ fetal liver hemopoietic precursors develop granulocytosis, indicates that certain Rel/NF-κB proteins are important in regulating granulocyte homeostasis *in vivo*.

C-terminal truncation mutations in Rel/NF-κB proteins

nfkb1$^{\Delta CT/\Delta CT}$ *mice* Mice lacking the portion of the *nfkb1* coding region that encompasses the C-terminal regulatory domain of p105 show a more severe phenotype than mice homozygous for an *nfkb1* null mutation (Ishikawa *et al.*, 1998). These mice (*nfkb1*$^{\Delta CT/\Delta CT}$ mice) develop splenomegaly, enlarged lymph nodes and have lymphocytic infiltrations of the lung and liver. They also exhibit heightened susceptibility to various opportunistic pathogens. The changes associated with dysregulated lymphocyte function appear to result from an increase in B-cell numbers that coincide with the hyperproliferative responsiveness of these cells to mitogens. In contrast, T cells from *nfkb1*$^{\Delta CT/\Delta CT}$ mice exhibit a weak reduction in proliferative capacity in culture and produce lower amounts of various cytokines after activation. Consistent with the evidence that the ankyrin repeats in the C terminus of p105 regulate cellular levels of p50, gel shift analysis indicates that the nuclear levels of p50 homodimers are elevated in the tissues of *nfkb1*$^{\Delta CT/\Delta CT}$ mice. These findings, together with those for the *nfkb2*$^{\Delta CT/\Delta CT}$ mice, reinforce the notion that tight regulation of Rel/NF-κB expression is crucial for normal cellular functions.

nfkb2$^{\Delta CT/\Delta CT}$ *mice* *nfkb2*$^{\Delta CT/\Delta CT}$ mice, which express the 52 kDa form encoded by *nfkb2* but lack the 100 kDa protein due to disruption of the *nfkb2* C-terminal coding region, appear normal at birth but develop multiple pathologies post-natally (Ishikawa *et al.*, 1997). These pathologies include gastric hyperplasia of the epithelial layer of the antrum, hyperkeratosis in the heart, lymphocytic infiltration in the lamina propia and hemopoietic abnormalities such as spleen and thymic atrophy, enlarged lymph nodes, and granulocytosis. The presence of increased numbers of lymphocytes in various tissues and lymph nodes is consistent with *nfkb2*$^{\Delta CT/\Delta CT}$ T cells being hyperresponsive to activation in culture. Tissues from *nfkb2*$^{\Delta CT/\Delta CT}$ mice that overexpress p52-containing nuclear complexes also upregulate several genes known to be controlled by Rel/NF-κB, including those encoding TNFα, I-CAM1 and ELAM-1. These findings indicate that p52 is normally involved in controlling the growth of gastric mucosal cells and mature peripheral lymphocytes, and that dysregulated expression of this transcription factor can lead to hyperproliferation, a finding consistent with the rearrangement of *nfkb2* in certain human lymphomas (Rayet and Gélinas, 1999).

c-rel$^{\Delta CT/\Delta CT}$ *mice* Deletion of the c-Rel C-terminal transactivation domains creates a protein still capable of forming homodimers or heterodimers with other NF-κB subunits and binding DNA, but unable to regulate transcription in a normal manner. Several months after birth, mice homozygous for this mutation (c-*rel*$^{\Delta CT/\Delta CT}$) develop hypoplastic bone marrow, splenomegaly, enlarged lymph nodes, and lymphoid hyper-

plasia (Carrasco *et al.*, 1998). Prior to the onset of dysregulated lymphocyte expansion, young c-*rel*$^{\Delta CT/\Delta CT}$ mice exhibit defects in B-cell activation and antibody synthesis and have increased susceptibility to *L. monocytogenes*, probably a result of reduced nitric oxide and GM–CSF production by macrophages. The molecular basis for the difference in the phenotypes of c-*rel*$^{-/-}$ and c-*rel*$^{\Delta CT/\Delta CT}$ mice most likely reflects in part functional compensation for c-Rel by other family members in the c-*rel*$^{-/-}$ mice versus the abnormal transcriptional activity of NF-κB-like complexes containing the C terminally-truncated c-Rel protein.

Null mutations for IκB proteins

ikba$^{-/-}$ *mice* IκBα is the major ubiquitous cytoplasmic inhibitor that is critical for regulating the rapid transient nuclear induction of Rel/NF-κB. Although the embryonic development of mice lacking IκBα appears to be normal, *ikba*$^{-/-}$ mice die 7–10 days post-natally, afflicted by severe widespread inflammatory dermitis and granulocytosis (Beg *et al.*, 1995; Klement *et al.*, 1996). Coincident with this phenotype, the expression of certain proinflammatory cytokines and factors associated with granulocyte recruitment, adherence and activation such as TNFα, G-CSF, MIP-2 and VCAM-1 is increased. However, not all genes known to be induced by Rel/NF-κB are upregulated in *ikba*$^{-/-}$ cells, underscoring the role of other transcriptional regulators in the activation of many Rel/NF-κB target genes. Furthermore, despite the absence of IκBα in all tissues, changes in the constitutive nuclear levels of Rel/NF-κB are cell type-dependent. For example, whereas an increase in constitutively nuclear p50/RelA and p50 homodimers was observed in *ikba*$^{-/-}$ thymocytes and splenocytes, the levels of constitutive Rel/NF-κB complexes were unchanged in *ikba*$^{-/-}$ embryonic fibroblasts. This finding indicates that IκBα is more important in regulating the cytoplasmic retention of Rel/NF-κB in hemopoietic than certain non-hemopoietic cells. However, in response to activation signals, the nuclear localization of Rel/NF-κB in *ikba*$^{-/-}$ fibroblasts is prolonged, indicating that IκBα is essential for post-induction repression of Rel/NF-κB.

In the absence of p50/p105, most *nfkb1*$^{-/-}$ *ikba*$^{-/-}$ mice survive significantly longer (3–4 weeks) before succumbing to the same inflammatory disease as *ikba*$^{-/-}$ mice (Beg *et al.*, 1995). The absence of p50 significantly reduced constitutive nuclear levels of Rel/NF-κB in thymocytes, suggesting that the constitive expression of Rel/NF-κB may be critical in the neonatal lethality of *ikba*$^{-/-}$ mice.

To assess the extent of functional redundancy between IκBα and IκBβ, two IκB proteins that share extensive structural and biochemical similarities but different patterns of expression, a 'knock-in' strategy was employed. This involved deleting the *ikba* coding region and replacing it with the *ikbb* gene, which was now under the transcriptional control of *ikba* regulatory sequences. In contrast to *ikba*$^{-/-}$ mice, these homozygous knock-in mice are normal (Cheng *et al.*, 1998). Consistent with the absence of inflammatory disease, the regulation of Rel/NF-κB is equivalent to that of wild-type mice. This indicates that IκBα and IκBβ have similar biochemical properties and that these two inhibitors have acquired different functions primarily through a differential pattern of expression.

bcl-3$^{-/-}$ *mice* Bcl-3, a distinct member of the IκB-like protein family expressed primarily in hemopoietic tissue and liver, selectively inhibits DNA binding by p50 homodimers, but can also transactivate κB-dependent gene expression in the presence of p52 homodimers (Bours *et al.*, 1993). *bcl-3*$^{-/-}$ mice develop normally, but exhibit defects in antigen-specific B- and T-cell responses when challenged with various pathogens (Franzoso *et al.*, 1997; Schwartz *et al.*, 1997). Both the Th1 response to *T. gondii* and the capacity to produce specific high affinity T cell-dependent IgG2a antibodies to influenza are impaired in *bcl-3* knockout mice. Moreover, consistent with the failure to mount a normal antibody response, follicular splenic B-cell numbers are reduced and germinal centre formation is severely diminished. The underlying basis of these defects may be due in part to impaired antigen-dependent priming, since naive T *bcl-3*$^{-/-}$ cells polyclonally activated *in vitro* are able to produce normal levels of Th1 cytokines such as IFN-γ (Franzoso *et al.*, 1997). Such a defect is consistent with the depletion and loss of specific splenic marginal zone macrophage populations associated with the disrupted splenic architecture observed in *bcl-3*$^{-/-}$ mice.

ikbe$^{-/-}$ *mice* IκBε is a highly-specialized inhibitor of Rel/NF-κB complexes and is expressed at high levels primarily in T cells of the thymus and spleen, and to a lesser extent, in lung, ovary and testis (Li and Nabel, 1997; Mémet *et al.*, 1999). However, IκBε-deficient mice are viable, fertile, and lacking in severe immune defects (Mémet *et al.*, 1999). The only detected alterations in *ikke*$^{-/-}$ mice are a 50% reduction in the number of CDD44^{-}CD25^{+} T cells and increased expression of certain immunoglobulin isotypes and some cytokines. Nevertheless, *ikke*$^{-/-}$ mice show a normal response to several pathogens (Mémet *et al.*, 1999). In part, the minimal effect of loss of IκBε may be due to compensatory up-regulation of IκBα and IκBβ expression in *ikke*$^{-/-}$ mice, and *ikke*$^{-/-}$ *ikka*$^{-/-}$ double mutant mice die earlier post-natally than single *ikka*$^{-/-}$ mice (Mémet and Israël, personal communication).

Null mutations for the IκB kinases

The principal IκB kinase (IKK) is a complex containing two related catalytic kinases, IKKα and IKKβ, and the regulatory protein IKKγ, which is involved in kinase activation (Mercurio and Manning, 1999). Despite the sequence similarity of IKKα and IKKβ, the analysis of single knockout mice for these kinases has established that Rel/NF-κB activation by proinflammatory cytokines is dependent largely on IKKβ, whereas IKKα induces Rel/NF-κB during skin and skeletal development in response to an unidentified morphogenetic signal(s).

ikka$^{-/-}$ *mice* Mice homozygous for an *ikka* null mutation die post-natally, afflicted with multiple morphological defects, the most striking of which is the encasement of the embryo in a shiny taut skin that prevents the emergence of fore- and hind-limbs (Hu *et*

al., 1999; Takeda *et al.*, 1999). Other defects include an absence of ears, truncation of the head and snout, and skeletal abnormalities affecting the vertebrae, sternum, skull and digital phalanges that arise from the absence or inappropriate fusion of bones. Although the limbs of *ikka*$^{-/-}$ mice appears as ill-formed protrusions, beneath the skin they are almost normal in size, although lacking defined digits. The failure of limbs to emerge during embryogenesis appears to be the result of a block in keratinocyte differentiation, while the lack of distinct digits is due to an absence of programmed cell death that normally occurs within the interdigital regions.

The finding that loss of IKKα in mice causes developmental defects, suggests that, as in flies (Govind, 1999), Rel/NF-κB proteins regulate genetic programs in vertebrates that are associated with development as well as with immunity. This conclusion, while seemingly at odds with an absence of developmental defects in mice lacking individual Rel/NF-κB subunits, may emphasize the redundant function of Rel/NF-κB proteins in mammals. Alternatively and certainly possible, the IKK complex, especially IKKα, phosphorylates substrates other than IκB or regulates additional signaling pathways. Nevertheless, evidence of a role for Rel/NF-κB factors in vertebrate limb and skin development has previously come from over-expressing mutant IκBα in chick embryos (Bushdid *et al.*, 1998; Kanegae *et al.*, 1998) and in the dermis of transgenic mice (Seitz *et al.*, 1998). The normal induction of Rel/NF-κB in *ikba*$^{-/-}$ embryonic fibroblasts in response to the pro-inflammatory cytokines TNFα or IL-1 occurs via IKKβ (Hu *et al.*, 1999; Takeda *et al.*, 1999), and this finding suggests that an unknown set of signals operating through IKKα is required for the induction of Rel/NF-κB during skin and skeletal development.

The Rel/NF-κB-regulated genes required for bone and skin differentiation that are activated via IKKα remain to be identified. Conservation of the vertebrate and invertebrate Rel/NF-κB pathways makes it highly likely that certain of the Dorsal-regulated genes important for embryonic pattern formation in flies will also be regulated by Rel/NF-κB in mammals. One such gene may be *twist*, the expression of which is reduced in *ikka*$^{-/-}$ embryos (Takeda *et al.*, 1999). The skull and bone defects in *ikka*$^{-/-}$ mice resemble the phenotypes seen in mice heterozygous for a null allele of *twist* and in people suffering from Saethre-Chotzen syndrome, an autosomal dominant disorder arising from mutations in *twist*. (Howard *et al.*, 1997). Other *ikka*$^{-/-}$ defects such as the lack of external ears, a partially split sternum and forked xiphoid resemble defects seen in mutations of various bone morphogenetic proteins (Hu *et al.*, 1999), suggesting that IKKα-dependent signals may regulate the localized expression of bone morphogenetic proteins.

ikkb$^{-/-}$ *mice* The loss of IKKβ leads to embryonic death between days E12.5 and E14.5 post-coitum (Li *et al.*, 1999; Tanaka *et al.*, 1999) and like *rela*$^{-/-}$ embryos, appears to result from fetal hepatocyte apoptosis. The conclusion that *ikkb*$^{-/-}$ and *rela*$^{-/-}$ mice die from a common defect is supported by the ability of TNF receptor I (Li *et al.*, 1999) and TNFα (Doi *et al.*, 1999) null mutants, respectively, to block the embryonic lethality associated with the loss of IKKβ and RelA. Consistent with a perturbation of TNFα signals leading to the death of *ikkb*$^{-/-}$ embryos, a weak induction of Rel/NF-κB in *ikkb*$^{-/-}$ mouse embryonic fibroblasts by TNFα establishes that proinflammatory cytokines induce Rel/NK-κB through IKKβ and not IKKα (Li *et al.*, 1999; Tanaka *et al.*, 1999). While the analysis of *in vitro* hemopoietic colony assays established from the fetal liver of *ikkb*$^{-/-}$ embryos indicates that hemopoiesis is normal in the absence IKKβ (Tanaka *et al.*, 1999), monocytic progenitors in the fetal liver of E12 *rela*$^{-/-}$ c-*rel*$^{-/-}$ embryos fail to differentiate *in vitro* due to apoptosis (Grossmann *et al.*, 1999). These findings support a model in which the activation of Rel/NF-κB in response to stimuli that promote monocyte differentiation operates via an IKKβ-independent pathway.

Transgenic mice

Rel/NF-κB transgenic mice

Despite the rearrangement and amplification of various Rel/NF-κB and IκB genes in human leukemias and lymphomas (Rayet and Gélinas, 1999), targeted over-expression of RelA (Perez *et al.*, 1995) or RelB (Weih *et al.*, 1996) in the thymocytes of transgenic mice does not lead to the development of thymic or peripheral T-cell abnormalities. However, this conclusion may be somewhat misleading in that over-expression of RelA does not result in an increase in its nuclear levels as there is a corresponding increase in endogenous IκBα, with which RelA forms a cytoplasmic complex. Over-expression of RelB, however, is linked to an increase in nuclear κB site binding activity (Weih *et al.*, 1996). Taken together, these findings indicate that IκBα differentially regulates RelA and RelB in thymocytes.

Transgenic mice over-expressing the v-Rel oncoprotein (Gilmore, 1999) in thymocytes develop T-cell leukemias (Carrasco *et al.*, 1996). The major nuclear v-Rel-containing DNA-binding complexes expressed in these tumors are v-Rel homodimers and v-Rel/p50 heterodimers. v-Rel-induced tumors develop faster in mice homozygous for the null allele of *nfkb1*, suggesting p50 retards v-Rel-mediated leukemogenesis (Carrasco *et al.*, 1996). However, over-expression of IκBα in v-Rel transgenic mice (Carrasco *et al.*, 1997), which selectively reduces the nuclear expression of v-Rel/p50 heterodimers but not v-Rel homodimers in thymocytes, retards leukemia onset and changes the characteristics of the disease. This suggests that the different v-Rel-containing dimers may play distinct roles in T-cell leukemogenesis.

IκB transgenic mice

Over-expression of IκBα$_m$ and IκBβ$_m$ Mutant IκBα and IκBβ proteins (IκBα$_m$ and IκBβ$_m$) that are no longer susceptible to signal-induced degradation have been expressed as transgenes to ablate the nuclear expression of Rel/NF-κB complexes in a developmental and tissue-specific fashion. Several groups have independently targeted these IκB 'super repressors' to the T-cell lineage using various T cell-specific

promoters (Attar *et al*., 1998; Boothby *et al*., 1997; Hettmann *et al*., 1999). While thymocyte development proceeds normally in the absence of individual Rel/NF-κB family members, there is a significant reduction in the number of peripheral $CD8^+$ T cells in mice expressing either $I\kappa B\alpha_m$ or $I\kappa B\beta_m$. The proliferative response of those remaining peripheral T cells or thymocytes to various mitogens, including cross-linking to the T-cell receptor, is impaired (Boothby *et al*., 1997; Hettmann *et al*., 1999), and $I\kappa B\beta_m$ transgenics display impaired T cell-dependent immune responses (Attar *et al*., 1997). Cross-linking of CD3 on double-positive (DP) thymocytes ($CD4^+CD8^+$) normally induces apoptosis, however DP thymocytes from $I\kappa B\alpha_m$ transgenic mice are resistant to this form of cell death (Hettmann *et al*., 1999). This suggests that Rel/NF-κB transcription complexes may promote thymocyte cell death under certain circumstances, a finding not inconsistent with the emerging model that these transcription factors can both promote and inhibit apoptosis (see Barkett and Gilmore, 1999).

In stratified epithelium, the mitotically active basal cells cease to divide and undergo terminal differentiation upon outward migration. The observation that NF-κB proteins are cytoplasmic in mitotically active basal cells but localize to the nucleus of differentiated supradermal cells suggested that NF-κB may be involved in the switch from proliferation to growth arrest and differentiation. Consistent with such as model, targeted expression of $I\kappa B\alpha_m$ to the epidermis of transgenic mice leads to epithelial hyperplasia (Setiz *et al*., 1998), while expression of transgenes for constitutively nuclear p50 or RelA mutants leads to an inhibition of epithelial cell growth (Seitz *et al*., 1998). These findings are consistent with the recent observation that IKKα-deficient mice, which fail to activate Rel/NF-κB in epithelial cells also exhibit a block in epithelial cell differentiation coupled with basal cell hyper-proliferation (Hu *et al*., 1999; Takeda *et al*., 1999).

Over-expression of Bcl-3 *bcl-3*, which is rearranged and over-expressed in chronic lymphocytic leukemia (Rayet and Gélinas, 1999), leads to an increase in p50 homodimer DNA-binding activity when over-expressed in transgenic mice (Caamaño *et al*., 1996). This finding contrasts with the transient over-expression of Bcl-3 in cell lines, where Bcl-3 inhibits DNA binding by p50 homodimers (Franzoso *et al*., 1992). Interestingly, over-expression of Bcl-3 in thymocytes does not induce T-cell leukemias. This indicates either that Bcl-3 is only oncogenic in certain cell lineages, that the stage in the transformation process when deregulated Bcl-3 expression occurs is critical, and/or that Bcl-3 over-expression requires additional mutational events to be oncogenic.

Concluding remarks

The information revealed by the mouse models described herein have provided many insights into the physiological roles of the Rel/NF-κB signal transduction pathway in mammals. Noteworthy examples of important, yet unexpected findings revealed by these mice include that morphogenetic and cytokine signals appear to be transmitted by IKKα- and IKKβ-dependent pathways respectively, that different combinations of Rel/NF-κB proteins are crucial in promoting B-cell differentiation and survival at different stages of development, and that Rel/NF-κB factors appear to play a more important anti-apoptotic role in B cells than in T cells. In the future these mice will prove to be crucial reagents for developing new cell models, for examining the roles of Rel/NF-κB in different diseases and for determining which target genes are regulated by particular Rel/NF-κB dimers.

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Immunology

Exhibit E

Pathways for self-tolerance and the treatment of autoimmune diseases

Christopher C Goodnow

Antigen delivers both immunogenic and tolerogenic signals to lymphocytes. The outcome of antigen exposure represents a complex integration of the timing of antigen binding with signals from many other immunogenic and tolerogenic costimulatory pathways. A road map of these signalling pathways is only beginning to be charted, revealing the mechanism of action and limitations of current immunotherapeutic agents and the points of attack for new agents. Ciclosporin and tacrolimus interfere with tolerogenic signals from antigen in addition to blocking immunogenic signals, thus preventing active establishment of tolerance. Corticosteroids inhibit a key immunogenic pathway, NFκB, and more specific inhibitors of this pathway may allow tolerance to be actively established while immune responses are blocked. New experimental therapies aim to mimic tolerogenic antigen signals by chronically stimulating antigen receptors with antigen or antibodies to the receptor, or aim to block costimulatory pathways involving CD40 ligand, B7, or interleukin 2. Obtaining the desired response with these strategies is unpredictable because many of these signals have both tolerogenic and immunogenic roles. The cause of autoimmune diseases has been determined for several rare monogenic disorders, revealing inherited deficiencies in tolerogenic costimulatory pathways such as FAS. Common autoimmune disorders may have a biochemically related pathogenesis.

Self-tolerance is an essential feature of the immune system, and works to protect tissue antigens from becoming targets of damaging immune responses during clearance of infection. The immune system normally exhibits exquisite specificity in distinguishing infectious antigens from self antigens. Vigorous antibody or T-cell responses are mounted against infectious antigens, whereas self antigens generally elicit only transient or weak responses even when incorporated into an infectious particle.

Adaptive immune responses start with the binding of antigen to antigen receptors on rare lymphocytes. The number and activity of these cells is then greatly expanded by clonal proliferation and differentiation. The response of individual lymphocytes is governed, however, by opposing immunogenic and tolerogenic signals, and the latter normally prevail for lymphocytes that bind self antigens. Disturbance in the natural balance between immunogenic and tolerogenic signals due to genetic factors can give rise to autoimmune disease. Progress in delineating these opposing signals provides opportunities to correct the primary disorder in autoimmune patients.

Counterbalancing immunogenic and tolerogenic signals

Two basic types of extracellular stimuli control lymphocyte growth and development (figure 1). The first is antigen signalling, through clone-specific antigen receptors. The second is costimuli, which encompasses a number of signals, through receptors that are not antigen specific. Importantly, particular antigen or costimuli signals are rarely obligately immunogenic or tolerogenic. Their timing and the way they are integrated at key checkpoints in lymphocyte development determines how a lymphocyte responds. Strongly immunogenic costimuli can shift the balance to immunity in the face of strongly tolerogenic antigen signals, and strongly tolerogenic costimuli can over-ride strongly immunogenic antigen signals. Deciphering the molecular logic behind this signal integration is the central challenge facing clinical manipulation of tolerance and immunity.

Immunogenic and tolerogenic antigen signals

Antigens transmit signals to lymphocytes by binding to B-cell receptors (surface immunoglobulin on B cells), and to T-cell receptors (TCRs) on T cells. B-cell receptors and TCRs signal through a cascade of protein tyrosine kinases and protein-lipid phosphorylation. Antigen transmits immunogenic or tolerogenic signals to lymphocytes through these receptors. Continuous

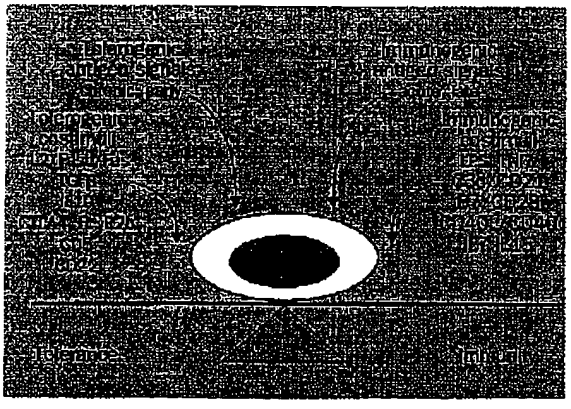

Figure 1: **Schematic diagram illustrating the balance of immunogenic and tolerogenic signals affecting lymphocyte responses to antigen**
LPS=lipopolysaccharide.

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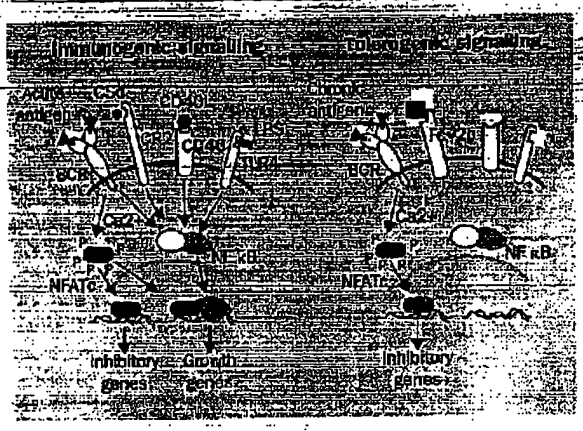

Figure 2: **Biochemically distinct signals underpin immunogenic and tolerogenic responses to antigen in B lymphocytes**
Immunogenic signalling occurs when antigen is encountered suddenly by mature B cells, and is augmented by co-clustering of complement C3d-receptor, CR2, and concurrent stimulation by CD40L from helper T cells or bacterial products such as lipopolysaccharide (LPS). One of the chief pathways activated by these signals is NFκB, a DNA binding protein family that moves to the nucleus once activated. In the nucleus, NFκB is pivotal to stimulating expression of many key B lymphocyte growth genes, promoting cell proliferation and antibody. The B-cell receptor also activates another DNA binding protein, NFATc, which moves to the nucleus after calcium-induced dephosphorylation and can work synergistically with NFκB. On its own, NFATc can activate inhibitory genes such as the inhibitory receptor CD72. Tolerogenic signalling occurs when antigen is encountered chronically, which results in inhibitory changes that diminish calcium signalling so that NFκB is no longer activated. Co-clustering of the receptor for IgG, FcγR2b, also inhibits immunogenic signalling to NFκB. Absence of costimuli such as CD40L or LPS is also critical to allow tolerogenic signalling to proceed in the absence of NFκB.

binding of antigen over several days, as is often the case for self antigens, usually transmits tolerogenic signals. By contrast, a sudden increase in receptor crosslinking, as occurs in most infections, tends to transmit immunogenic signals. Binding of antigen during immature lymphocyte formation in bone marrow or thymus, as occurs for many self antigens but few infectious antigens, tends to be tolerogenic.[1] Immunogenic signals are favoured when antigen is first encountered after lymphocytes have matured and reached the secondary lymphoid tissues, where infectious antigens tend to be trapped.

Immunogenic and tolerogenic antigens elicit different biochemical signals within lymphocytes[2] (figure 2). These biochemical differences provide opportunities to develop immunosuppressants that mirror these different signal patterns. In mature B lymphocytes, tolerogenic signalling by antigen elicits a smaller calcium response than immunogenic antigen. The calcium concentration achieved with tolerogenic signals is enough to activate the nuclear factor of activated T cells (NFATc) but insufficient to activate the nuclear factor kappa binding molecule (NFκB). NFATc and NFκB are DNA binding transcription factors that promote expression of different sets of genes. NFAT is essential for turning on lymphocyte inhibition as well as activatory genes, whereas NFκB is more purely immunogenic, because it is essential for inducing genes necessary for B and T cell proliferation and antibody production. As a result, a different pattern of gene expression is established by tolerogenic and immunogenic exposures to the same antigen.[3]

Deficiency of the NFκB transcription factor, c-rel, abolishes both T and B cells' immunogenic responses to antigen.[4] The inherited immunodeficiency syndrome, X-linked agammaglobulinaemia, is caused by defects in Bruton's tyrosine kinase (BTK), an intracellular enzyme that is essential for immunogenic signalling to NFκB by B-cell receptors.[5] Tolerogenic signalling to antigen remains intact or enhanced in BTK-defective B cells. This selective role in immunogenic signalling might explain the powerful suppression of systemic lupus in NZB/W mice when defects in BTK are introduced by breeding.[6] The selective role of the BTK/NFκB pathway in immunogenic signalling to antigen thus makes it an attractive target for new immunosuppressive drugs.

Immunogenic costimuli from microorganisms

Costimuli arise from many sources in the lymphocyte microenvironment. Perhaps the only purely immunogenic costimuli come from conserved components of infectious microorganisms. The lipopolysaccharide (LPS) moiety of bacterial cell walls and DNA rich in the dinucleotide, CpG from bacteria both activate the NFκB pathway in lymphocytes through surface receptors of the Toll-like receptor (TLR) family[7,8] (figure 2). These immunogenic costimuli also signal lymphocytes indirectly by activating antigen presenting cells—dendritic cells, macrophages, and B cells, to produce additional immunogenic costimuli such as the T cell activating cell surface protein B7 (CD80) and the inflammatory cytokine tumour necrosis factor alpha (TNFα). Bacterial adjuvants have been explored as experimental therapeutics to promote immunogenic responses to autoantigens on tumour cells but give rise to other undesirable inflammatory effects. Their effect may be more specifically emulated by activating dendritic cells bearing tumour antigens in vitro and giving these cells to the patient.

Costimuli from stressed and dying cells

Cell death through apoptosis occurs physiologically in healthy tissues without inflammation or immunogenicity. Engulfment of apoptotic cells by tissue macrophages, dendritic cells, or fibroblasts elicits signals through the phosphatidylserine receptor that promote synthesis of the tolerogenic cytokine, transforming growth factor beta (TGFβ; figure 3) and inhibit production of the immunogenic cytokine TNFα.[9] By contrast, pathological cell death by necrosis links antigens with immunogenic costimuli. Necrotic cells, and antigens released from necrotic or stressed cells complexed with the heat-shock proteins, Hsp96 and Hsp70, activate dendritic cells to express immunogenic costimuli including B7 and TNFα.[10–12] In patients and animals models with developing neoplasms, increased production of these immunogenic costimuli through cell dysplasia and necrosis may account for the frequent detection of subclinical autoantibodies and for the less frequent paraneoplastic autoimmune syndromes. The latter might simply reflect rare clinical manifestations of common autoimmune responses to dysplastic tumour cell autoantigens, as a result of chance reactivity of the autoantibodies with a vital cell receptor. Likewise, cell stress and dysfunction in specific organs, such as the pancreatic beta cell, may be an immunogenic costimulus for autoimmunity.

Dual role of the complement system

Activation of the serum complement system by foreign cells or particles produces powerfully immunogenic costimuli, partly by covalently tagging the infectious antigens with the complement cleavage product C3d.[13] C3d signals immunogenically to B lymphocytes, through the complement C3d receptors, CR1 and CR2 (CD21

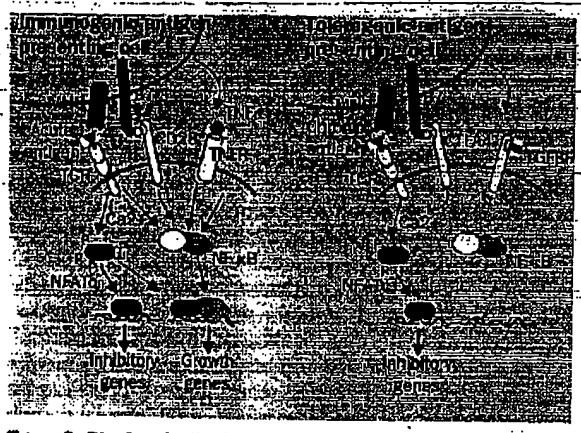

Figure 3: Biochemically distinct signals underpin immunogenic and tolerogenic responses to antigen in T lymphocytes

Immunogenic signalling occurs when antigen peptide and MHC complexes are encountered suddenly by mature T cells, and is augmented by concurrent stimulation by B7 molecules engaging CD28, or binding of TNFα. One of the chief pathways activated by these signals is NFκB, a DNA binding protein family that moves to the nucleus once activated. In the nucleus, NFκB is pivotal to stimulating expression of many key T lymphocyte growth genes, promoting cell proliferation and inflammatory cytokines. The TCR also activates another DNA binding protein, NFATc, which moves to the nucleus after calcium-induced dephosphorylation and can work synergistically with NFκB. On its own, NFATc can activate inhibitory genes such as the death receptor ligand, FasL. Tolerogenic signalling occurs when antigen is encountered chronically, which results in inhibitory changes that diminish calcium signalling so that NFκB is no longer activated. CD28 is downregulated and an inhibitory receptor for B7, CTLA4, is upregulated. Concurrent stimulation by TGFβ inhibits expression of lymphocyte growth genes.

and CD35), when a C3d-tagged antigen causes clustering of these receptors with the B-cell receptors (figure 2). Complement components C1, C2, C4, and the CR1/2 complement receptors are also important for delivering tolerogenic signals, since inherited deficiencies of these elements in human beings and mice are associated with susceptibility to autoimmune disease. C1q deficiency leads to an inability to clear apoptotic cells efficiently, and this may either diminish the tolerogenic signals elicited by physiological cell corpses or allow them to become immunogenic.[14]

Dual role of the B7 system

Cell surface proteins of the B7 family, displayed on antigen presenting cells such as macrophages, dendritic cells, and B lymphocytes, deliver immunogenic costimuli to T cells by signalling through the CD28 and inducible costimulator (ICOS) receptors[15,16] (figure 3). The B7.1 and B7.2 proteins are induced on antigen presenting cells by other immunogenic costimuli, such as LPS, necrotic cells, or immunogenic antigen receptor signals in B cells, creating a cascade of immunogenic signals. Immunosuppressive therapy aimed at blocking the immunogenic effects of B7.1 and B7.2, notably the recombinant protein antagonist CTLA4-Ig, has been shown to improve the symptoms of psoriasis.

B7/CD28 costimuli are tolerogenic in other contexts, notably in immature thymocytes where they enhance clonal deletion. The B7/CD28 pathway also promotes tolerance by signalling the formation of regulatory CD4+CD25+ T cells that may be required for tolerance to tissue antigens.[17] B7.1 and B7.2 proteins also transmit tolerogenic signals to T cells by engaging another receptor, CTLA4, that is present at very low levels in resting T cells and substantially increased by chronic antigen signals[18] (figure 3). The importance of CTLA4 as a brake to the system is shown by the lethal inflammatory and lymphoproliferative disorder that occurs in CTLA4-deficient mice, and by the augmented autoimmune responses to melanoma antigens that occur when CTLA4 is blocked with antibodies.

Dual role of TNFα family of proteins and receptors

Activation of T cells and other innate or adaptive immune cells elicits an important and growing class of immunogenic and tolerogenic costimuli related to the cytokine, TNFα. TNFα itself has a pleiotropic effect on immune responses and inflammatory cells.[19–21] In some contexts, TNFα promotes self-tolerance and CD8 T cell deletion, whereas in others TNFα promotes T cell activation and autoimmune disease. Inherited deficiencies in TNFα or its receptors in mice results in poor cytotoxic T-cell-mediated resistance to certain viruses and inability to form follicular dendritic cells needed for humoral immunity. Symptoms of rheumatoid arthritis improve after blocking TNFα with antibodies or recombinant protein antagonists, indicating that production of this cytokine by T cells in the synovium has a key inflammatory role.[22]

CD40-ligand (CD40L) and Fas-ligand are two proteins related to TNFα with essential regulatory functions. Both are membrane-bound proteins displayed on T cells following T-cell receptor signals. CD40L engages its receptor, CD40, on B cells and dendritic cells to activate immunogenic responses through the NFκB pathway.[23] The importance of CD40L as an immunogenic costimulus is shown in children and mice with inherited CD40L deficiency, the X-linked hyper-IgM syndrome, where there is an absence of IgG antibody responses and defective T-cell immunity. Experimental therapies based on blocking the immunogenic effects of CD40L on B cells and dendritic cells with antibodies showed spectacular promise in animal models, notably achieving long-term allograft tolerance in primates.[24] Clinical trials in human beings have been suspended, however, because of thromboembolic complications in a subset of participants.

CD40L also seems to have an important tolerogenic role, since CD40L-deficient children are also commonly affected by autoimmune disease. CD40L is needed as a tolerogenic signal for B cells to increase expression of Fas (CD95), the receptor for FAS-L.[25] FAS itself transmits a potent tolerogenic costimulus by triggering the death and deletion of self-reactive B and T lymphocytes. The importance of the FAS pathway is seen by the systemic Autoimmune Lymphoproliferative Syndrome (ALPS) in human beings and mice with inherited deficiencies in FAS-L, FAS, or the downstream protease Caspase-10.[21,26]

Inhibitory co-receptors

This is a rapidly growing class of receptors that transmit inhibitory or tolerogenic costimuli to lymphocytes functions by recruiting protein tyrosine or lipid phosphatases. The prototype for this family is the low affinity receptor for IgG, FcγR2b, on B cells.[27] Antigen and antibody complexes cause the antigen receptors to cluster with FcγR2b, preventing B cell activation by otherwise immunogenic antigens and, instead, triggering death and deletion of the B cells (figure 2). This mechanism is believed to explain the tolerogenic effect of anti-RhD prophylaxis, in which small amounts of IgG anti-RhD antibodies given to Rh-negative mothers prevent maternal antibody responses to fetal RhD antigen.

Cytokines

Cytokines deliver both immunogenic and tolerogenic costimuli to lymphocytes. This balance is well illustrated by interleukins 2, 7, and 15.[21,28-30] All three signal T and B cells through multisubunit receptors that share a common gamma chain (γc). Inherited deficiency of the γc subunit accounts for X-linked Severe Combined Immunodeficiency, characterised by lack of naïve or memory T and B cells. IL-7 delivers essential costimuli through γc that promote naïve T and B cell formation in thymus and bone marrow and promote survival of naïve T cells in the lymph nodes. Similarly, growth and persistence of memory CD8 T cells is promoted primarily by IL-15. By contrast, the essential function of IL-2 in vivo is to deliver a tolerogenic costimulus, despite its original discovery in tissue culture as a so-called T-cell growth factor. Mice lacking IL-2 or the unique IL-2 receptor alpha subunit develop a severe T cell lymphoproliferative disease with numerous autoantibodies. IL-2 sensitises T cells to receive tolerogenic signals by the Fas receptor system, and may also be required to sustain a tolerogenic subset of CD4+CD25+ regulatory T cells.

Transforming growth factor beta (TGF-β) delivers an important tolerogenic signal to lymphocytes, and mice lacking this cytokine rapidly develop a lethal syndrome of lymphocyte hyperactivity and autoantibodies.[31] TGF-β inhibits the entry of lymphocytes into the cell cycle, and thus might establish a high tolerogenic threshold against which immunogenic signals from antigen and costimuli must work to initiate lymphocyte responses. The early response to immunogenic antigen is differentiated from tolerogenic antigen responses in part by rapid downregulation of inhibitory transcription factors in the former.[3] TGF-β seems likely to establish these inhibitory factors in quiescent and tolerised lymphocytes. TGF-β production by macrophages is induced by recognition and engulfment of cells that have died by physiological (non-inflammatory) apoptosis.[9] Macrophages, dendritic cells, and T cells making TGF-β seem to promote tolerance to self and foreign antigens in the eye, lung, and gut.[32,33] Linking antigen signals with TGF-β signals may be the basis for the experimental phenomenon of oral tolerance. Clinical trials are underway aimed at preventing type 1 diabetes or ameliorating multiple sclerosis by inducing oral tolerance to pro-insulin or myelin basic protein.

Integration of tolerogenic and immunogenic signals at different steps in the immune response

Integration and timing of antigen signals and costimuli occur at numerous checkpoints in lymphocyte development. These checkpoints are placed all along the developmental pathway, from those that delete newly formed B or T cells in the bone marrow and thymus through to those that abort the formation of terminally differentiated plasma cells or killer cells. Lymphocytes integrate antigen signals and costimuli very differently from one checkpoint to another, because expression of receptors and their intracellular response machinery change during development. The multiplicity of checkpoints exists presumably for two main reasons. First, no single mechanism can adequately ensure tolerance to all self antigens. Second, the existence of multiple mechanisms balances the need for tolerance against the need to use cells that crossreact between self and foreign antigens for rapid immunity against infection.[34]

Clonal deletion in central lymphoid tissues

In the bone marrow and thymus, antigens that cluster antigen receptors rapidly and avidly—which would be immunogenic for a mature lymphocyte—are almost exclusively tolerogens for newly-formed B and T cells.[35] The basis for the tolerogenic response of immature lymphocytes seems to be a result of many things: differences in the second messengers elicited by antigen receptors in immature cells, differences in the set of genes that can be triggered by second messengers, and presence of tolerogenic costimuli in the bone marrow and thymus microenvironments. Immature thymocytes are triggered to die even when antigen signals are linked with costimuli such as B7/CD28 that would be immunogenic to mature T cells. In immature B cells, continuous B-cell receptor engagement with strongly crosslinking self antigens, such as DNA or surface antigens on haematopoietic cells, delivers a tolerogenic signal that immediately arrests the cell's maturation and leads to clonal deletion within 1–3 days. Some of these arrested cells reach the spleen before dying, but they are extraordinarily refractory or anergic to immunogenic costimuli such as LPS and CD40. Particular combinations of immunogenic costimuli, such as CD40 and IL-4 from helper T cells, may be able to over-ride the powerfully tolerogenic signals from self antigen in these situations and break tolerance at this point.

Only a subset of self antigens are nevertheless present in sufficient quantity in the thymus and bone marrow to trigger clonal deletion. There is simply not enough antigen to signal deletion for most clones which recognise antigens present in trace quantities in the circulation or which are restricted to other tissues, such as the pancreatic islets, the brain, or the thyroid. Other mechanisms normally ensure tolerance to these antigens.

Clonal anergy

Self antigens that are present in lesser amounts in the bone marrow or thymus, or that cluster antigen receptors less avidly, can signal repeatedly to B and T cells without attaining the threshold needed to trigger arrest and death.[3,36] This constant "tickling" of antigen receptors by self antigens nevertheless transmits tolerogenic signals, activating feedback mechanisms that render the cell more refractory or anergic to immunogenic antigen signals. Anergy mediates B cell tolerance to self DNA and chromatin, and CD4 T cell tolerance to systemic and organ-specific antigens. In both B and T cells, anergy seems to involve a selective weakening of the connections between antigen receptors and the NFκB and JNK intracellular signalling pathways. Signalling through other intracellular pathways such as NFAT remains intact, so that a different set of tolerogenic genes is induced and immunogenic cell growth genes controlled by NFκB and JNK are not called into action. The weakening of connections to NFκB and JNK raises the threshold of immunogenic signalling needed to trip a self-reactive cell into multiplication. In B cells, a sudden burst of very avid antigen receptor clustering, or strong signals from LPS or CD40, allow sufficient signalling to the NFkB pathway to break anergy and drive the cell growth cycle.

Clonal deletion and regulation in peripheral lymphoid tissues

In addition to anergy, a series of peripheral deletion mechanisms catch self-reactive cells that reach the spleen, lymph nodes, and other organs.[35] These peripheral tolerance checkpoints act by shortening

lymphocyte lifespan, inhibiting lymphocyte migration and recirculation, or causing rapid cell death in germinal centres or liver. These peripheral processes are for the most part poorly understood in biochemical terms, with the exception of the peripheral elimination of autoreactive B and T cells through the Fas cell death pathway.[21,25,26,37]

Pathogenesis of autoimmune diseases

How does autoimmune disease arise? Given the range of self-tolerance processes, and the difficulty eliciting or maintaining autoimmune responses by deliberate means (for example in medical and veterinary efforts to achieve immunological contraception or castration), it is reasonable to ask how tolerance to one or more self antigens fails in many people. The reason is as yet unknown, except for the rare patients with inherited monogenic disorders such as ALPS and X-linked hyper-IgM.

Most of the common autoimmune diseases also have an important inherited element, contributing as much as 50% of the population risk, and particular types of autoimmune diseases thus cluster in families. This inherited susceptibility is nevertheless complex involving combinations of many different gene alleles.[38] The strongest contributions are made by particular haplotypes of the major histocompatibility complex (MHC) and specific HLA alleles within the MHC, whose products present antigen peptides to T cells. Exactly how particular MHC alleles predispose to autoimmunity is not yet established, and one can hypothesise too much or too little presentation of particular antigens by products of suceptible HLA alleles. Correlations between autoimmune susceptibility and many other chromosomal regions have been found in human beings and mice, but the complexity of the inheritance pattern has made it challenging to identify the non-MHC susceptibility genes.

Four basic kinds of defect may potentially give rise to autoimmune disease, either alone or in combination. A central challenge for clinical immunology will be to define which of these faults actually applies for individual patients, since the nature of the deficit will determine the success or failure of emerging therapeutic strategies.

Insufficient tolerogenic signalling from antigen

In order for deletion, anergy, or regulation to be triggered by tolerogenic signalling through antigen receptors, a sufficient number of receptors must be engaged on self-reactive cells. Autoantigens that are only present in trace amounts in the lymphatic tissues will not achieve this signalling threshold on any but the very highest affinity clones. If the autoantigen is highly expressed in extralymphatic sites, as is the case for insulin, thyroglobulin, myelin proteins, skin basement proteins, and type 2 collagen, these concentrated depots of autoantigen might suddenly deliver an acute immunogenic stimulus to self-reactive cells that chance to migrate into these sites. This situation seems to be the case for B cells and some CD8 T cells.[39,40] For CD4 T cells recognising such antigens, there seems to be some autoantigen encountered in lymphatic sites that might induce anergy and regulatory cells.[35]

Several susceptibility genes for type 1 diabetes may act by further diminishing this already limiting pathway for tolerogenic autoantigen presentation. Diabetes-susceptible MHC Class II alleles in human beings and mice seem less efficient at presenting antigens, potentially explaining the heightened risk of autoimmunity in individuals who are homozygous for these alleles.[41] A variant allele of the insulin gene associated with type 1 diabetes susceptibility is expressed at lower levels in the thymus, potentially lessening presentation and education of regulatory T cells to this antigen.[42]

If the primary lesion in individuals susceptible to type 1 diabetes and other organ-specific diseases is simply one of inadequate tolerogenic signals from the target self antigens, then delivering more of these antigens in a tolerogenic form is a rational strategy. Obviously, this approach has the risk of inducing autoimmunity if the self antigen is delivered in an immunogenic form in some individuals, either due to the way the antigen is presented, to presence of immunogenic costimuli, or to presence of primed or memory lymphocytes that may be more refractory to tolerogenic signals. A better understanding of the molecular integration of tolerogenic and immunogenic signals may be critical to the success of specific vaccines against diabetes and other autoimmune diseases.

Insufficient tolerogenic signals from autoantigen might also explain shortcomings of the immunosuppressive drugs, ciclosporin and tacrolimus (FK506). These drugs block the calcium/calcineurin/NFAT signalling pathway. This pathway is continually activated by self antigen in anergic B and T cells, and is important for inducing tolerogenic costimuli on lymphocytes such as CD72 and FAS-L. Interference with these actively tolerogenic signals might explain the systemic autoimmune disorders that can occur after cessation of the drug, and might account for the inablility to achieve long-term allograft acceptance with these agents.[3] The presence of circulating autoantibodies may compound autoimmunity in systemic lupus by blocking the presentation of tolerising autoantigens to B cells.[43]

Too much imunogenic signalling from antigen

Sudden presentation of viral or bacterial antigen in a highly crosslinked, immunogenic form, and associated with immunogenic costimuli produced by the infection, can provoke immune responses from T or B cells that crossreact with the microbial antigen and a self antigen. In animal models, this route can activate ignorant T and B cell clones that, through a combination of lower affinity receptors and limiting self-antigen presentation, had not received appreciable tolerogenic signals.[39,40,44,45] Moreover, if the immunogenic antigen stimulus is very strong, such as occurs with highly multimeric forms of antigen for B cells, the stimulus can overcome strongly tolerogenic antigen signals to break anergy[46,47] or over-ride clonal deletion.[48] Whereas a microbial trigger is postulated to be the cause of a number of common autoimmune diseases, perhaps the best established clinical example is the immunopathological damage of heart valves by antibodies that crossreact between valvular antigens and streptococcal M protein.

Interestingly, the self-reactive components of crossreactive responses are usually transient and lack memory in most experimental and practical situations in which tolerance is transiently broken by immunogenic delivery of self and foreign antigens. This phenomenon is a longstanding problem for medical and veterinary efforts to achieve immunocontraception and immunocastration, in which the autoantibody titres to pregnancy or sex hormones fall prematurely in the face of heightened titres to the foreign carrier proteins. Susceptibility to full-blown autoimmune disease might therefore require that a crossreactive trigger be coupled with deficits in the tolerogenic costimuli that normally create an inhibitory feedback on self antigen responses.

Deficiency of tolerogenic costimuli

Many of the rare systemic autoimmune disorders that are inherited as monogenic traits in human beings and mice arise from deficiencies of tolerogenic costimuli. The clearest example is human autoimmune lymphoproliferative syndrome (ALPS), which results from partial or complete deficiency in signalling by the death receptor FAS.[23] Similarly, deficiency of CD40L in X-linked hyper-IgM syndrome is commonly accompanied by autoimmune disorders that might reflect the need for CD40L to induce Fas on self-reactive B cells.[25] The monogenic autoimmune disorders listed above are clinically distinct from the common forms of autoimmune disease, but they illustrate the essential and non-redundant role of tolerogenic costimuli as brakes on autoimmunity. Common autoimmune disorders probably arise from collections of more subtle gene variants that collectively diminish the same tolerogenic pathways. In support of this notion, the type 1 diabetes susceptibility gene in the NOD mouse, *Idd3*, seems to be a variant form of IL-2 that may reduce the in-vivo efficacy of this tolerogenic costimulus.[26]

Too much immunogenic costimuli

There are many artificially engineered animal models where overexpression of immunogenic costimuli predisposes to autoimmune disease. For example, mice that overexpress TNFα, B7.1, IL-2, or IL-4 on pancreatic islet β-cells are predisposed to type 1 diabetes.[59] Cell death by necrosis releases antigens complexed with immunogenic costimuli, notably the heat-shock proteins HSP70 and HSP96, and necrotic cells activate dendritic cells. An increase in these tolerogenic immunogenic costimuli might explain the immunogenicity of dysplastic tumours that are commonly manifested by the appearance of subclinical autoantibodies to tumour antigens and by paraneoplastic autoimmune syndromes. Along similar lines, the inability to clear dead cells or chromatin might provoke systemic lupus in people with complement C1q deficiency.[14]

Targets for current and future therapy of autoimmune disease

The unfolding of the human genome project will accelerate assembly of a molecular map of immunogenic and tolerogenic signalling pathways. Translating this knowledge into cures for common autoimmune diseases will involve researchers addressing two key challenges. First, we must develop ways to diagnose the underlying cause of autoimmune disease in individual patients. There is probably little to be gained by giving an exogenous source of tolerogenic costimuli such as TGF-β or Fas-ligand to patients with an underlying problem further downstream in the receptors or signal-transduction pathways for these molecules. Methods for obtaining a genetic fingerprint of thousands of immunologically relevant genes will soon become available, and these might provide a way to shortlist the likely pathogenic deficits in individuals. Confirmation will probably require diagnostic biomarkers or specific assays for discrete immunogenic or tolerogenic pathways that can be done on blood samples.

The second critical element is development of protein or small molecule therapeutics that target critical pathways, either augmenting tolerogenic pathways or blocking immunogenic ones. Some of the best current agents for treating systemic autoimmune diseases, such as glucocorticoids, choloroquine, and gold compounds, seem to work by blocking the immunogenic NFκB pathway.[31] Improvements on these agents depend on narrowing the action to specific subsets of lymphocytes, and avoiding the undesirable metabolic effects of glucocorticoids. To cure fully developed autoimmunity, drug targets will need to come from understanding why memory T and B cells are more refractory to tolerogenic signals and why they are less dependent upon immunogenic costimuli.

Engineered proteins and antibodies aimed at blocking specific immunogenic costimuli upstream of NFkB, notably antibodies against TNFα,[32] CD40L,[24] and the blockers of B7 ligands of CD28, have shown great promise in mouse models and in clinical trials as agents to treat rheumatoid arthritis or establish transplantation tolerance. These strategies may be most effective in individuals with healthy tolerogenic signalling, such as patients undergoing organ transplantation, where the underlying defect is known to be an excess of immunogenic antigen and immunogenic costimuli. In this case, temporarily blocking the immunogenic signals selectively should allow tolerogenic antigen and costimuli to establish an active, reinforcing state of tolerance that persists when blocking therapy is stopped. However, if inherited deficits in tolerogenic signalling prevent restoration of tolerance during a brief window of blocking therapy, it will be necessary to continue the immunogenic blockers for long periods, even though there are many complications associated with long-term immunosuppression.

An attractive notion is the idea of so-called negative vaccines; vaccines that could deliver specific antigens in a way that augments tolerogenic rather than immunogenic signalling. In animal models, delivering low amounts of antigen by the mucosal route, either ingested or nasally, can act as a potent tolerogen. This process might perhaps work by linking the antigen with the tolerogenic costimulus, TGF-β, which features in mucosal immune responses.[32,33] The first clinical trial of oral tolerance was unsuccessful, pointing to the need to understand better the mechanisms involved and to develop ways to achieve more reliable linkage between tolerogenic antigen and suitable tolerogenic costimuli. Likewise, rational molecular strategies are needed to improve the success rate of empirical regimes for desensitising allergic reactions to pollens and venoms and to restore tolerance to blood products such as clotting factor VIII.

The one shining example of a successful tolerogenic vaccine is the prevention of erythroblastosis fetalis in Rh-antigen incompatible pregnancies by giving small amounts of anti-RhD antibody. The antibody converts an immunogen (fetal red cells) into a tolerogen by recruiting a tolerogenic costimulus, FcγR2b. The wide success and cost-benefit of this simple method is an example of how it should become possible to shift the balance back towards tolerance in an antigen specific way for many autoimmune diseases. The key lies in understanding the molecular interplay between immunogenic and tolerogenic pathways and having a way to forge the desired tolerogenic connections in specific lymphocyte clones.

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Editorial Review

Exhibit F

Anti-inflammatory actions of glucocorticoids: molecular mechanisms

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1. Glucocorticoids are widely used for the suppression of inflammation in chronic inflammatory diseases such as asthma, rheumatoid arthritis, inflammatory bowel disease and autoimmune diseases, all of which are associated with increased expression of inflammatory genes. The molecular mechanisms involved in this anti-inflammatory action of glucocorticoids is discussed, particularly in asthma, which accounts for the highest clinical use of these agents.

2. Glucocorticoids bind to glucocorticoid receptors in the cytoplasm which then dimerize and translocate to the nucleus, where they bind to glucocorticoid response elements (GRE) on glucocorticoid-responsive genes, resulting in increased transcription. Glucocorticoids may increase the transcription of genes coding for anti-inflammatory proteins, including lipocortin-1, interleukin-10, interleukin-1 receptor antagonist and neutral endopeptidase, but this is unlikely to account for all of the widespread anti-inflammatory actions of glucocorticoids.

3. The most striking effect of glucocorticoids is to inhibit the expression of multiple inflammatory genes (cytokines, enzymes, receptors and adhesion molecules). This cannot be due to a direct interaction between glucocorticoid receptors and GRE, as these binding sites are absent from the promoter regions of most inflammatory genes. It is more likely to be due to a direct inhibitory interaction between activated glucocorticoid receptors and activated transcription factors, such as nuclear factor-κB and activator protein-1, which regulate the inflammatory gene expression.

4. It is increasingly recognized that glucocorticoids change the chromatin structure. Glucocorticoid receptors also interact with CREB-binding protein (CBP), which acts as a co-activator of transcription, binding several other transcription factors that compete for binding sites on this molecule. Increased transcription is associated with uncoiling of DNA wound around histone and this is secondary to acetylation of the histone residues by the enzymic action of CBP. Glucocorticoids may lead to deacetylation of histone, resulting in tighter coiling of DNA and reduced access of transcription factors to their binding sites, thereby suppressing gene expression.

5. Rarely patients with chronic inflammatory diseases fail to respond to glucocorticoids, although endocrine function of steroids is preserved. This may be due to excessive formation of activator protein-1 at the inflammatory site, which consumes activated glucocorticoid receptors so that they are not available for suppressing inflammatory genes.

6. This new understanding of glucocorticoid mechanisms may lead to the development of novel steroids with less risk of side effects (which are due to the endocrine and metabolic actions of steroids). 'Dissociated' steroids which are more active in transrepression (interaction with transcription factors) than transactivation (GRE binding) have now been developed. Some of the transcription factors that are inhibited by glucocorticoid, such as nuclear factor-κB, are also targets for novel anti-inflammatory therapies.

INTRODUCTION

Glucocorticosteroids suppress inflammation in a wide variety of diseases, including allergic diseases, rheumatoid arthritis, inflammatory bowel disease and autoimmune diseases. Indeed they are often the most effective therapy available and their use is limited only by systemic side effects. The most widespread use of glucocorticoids is in asthma and inhaled glucocorticoids have revolutionized treatment and now become

Key words: asthma, glucocorticoid, inflammation, transcription factor.

Abbreviations: AP-1, activator protein-1; CBP, CREB binding protein; COX-2, inducible cyclo-oxygenase; CREB, cyclic AMP responsive element binding protein; FGR, familial glucocorticoid resistance; GCR, glucocorticoid resistant; GCS, glucocorticoid sensitive; GM-CSF, granulocyte–macrophage colony-stimulating factor; GR, glucocorticoid receptor; GRE, glucocorticoid response elements; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; iNOS, inducible nitric oxide synthase; NF-AT, nuclear factor of activated T-cells; NF-κB, nuclear factor-κB; NO, nitric oxide; PLA_2, phospholipase A_2; STAT, signal transducer and activator of transcription; TNF-α, tumour necrosis factor-α.

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the mainstay of therapy for patients with chronic disease [1]. There have been important advances in our understanding of how glucocorticoids suppress inflammation and this may point the way to the development of improved glucocorticoids and more specific therapies in the future [2,3]. In this review I have focused on asthma as an example of an inflammatory disease that is suppressed by glucocorticoids. Asthma is the commonest inflammatory disease world-wide and accounts for by far the greatest amount of prescribed glucocorticoids.

GLUCOCORTICOID RECEPTORS

Glucocorticoids exert their effects by binding to a glucocorticoid receptor (GR) localized in the cytoplasm of target cells. There is a single class of GR that binds glucocorticoids, with no evidence for subtypes of differing affinity in different tissues. Recently a splice variant of GR, termed GR-β, has been identified that does not bind glucocorticoids but binds to DNA and may therefore potentially interfere with the action of glucocorticoids [4]. The structure of GR has been elucidated using site-directed mutagenesis, which has revealed distinct domains [5]. The glucocorticoid binding domain is at the C-terminal end of the molecule and in the middle of the molecule are two finger-like projections that interact with DNA. Each of these 'zinc fingers' is formed by a zinc molecule bound to four cysteine residues (Figure 1). An N-terminal domain (τ_1) is involved in transcriptional *trans*-activation of genes once binding to DNA has occurred and this region may also be involved in binding to other transcription factors. This is the least conserved part of the molecule between individuals and between species. Deletion analysis has demonstrated a 41-amino-acid core at the C-terminal end of the τ_1 domain that is critical for *trans*-activation. In human GR there is another *trans*-activating domain (τ_2) adjacent to the glucocorticoid binding domain and this region is also important for the nuclear translocation of the receptor. GR is phosphorylated (predominantly on serine residues at the N terminal), but the role of phosphorylation in glucocorticoid actions is not yet certain.

The inactivated GR is bound to a protein complex (approx. 300 kDa) which includes two molecules of 90 kDa heat shock protein (hsp90), a 59 kDa immunophilin protein and various other inhibitory proteins. The hsp90 molecules act as a 'molecular chaperone',

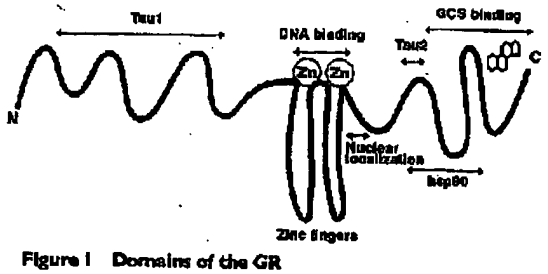

Figure 1 Domains of the GR

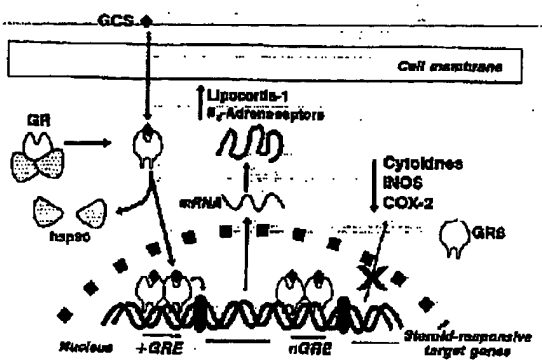

Figure 2 Classical model of glucocorticoid action

The glucocorticoid enters the cell and binds to a cytoplasmic glucocorticoid receptor (GR) that is complexed with two molecules of a 90 kDa heat shock protein (hsp90). GR translocates to the nucleus where, as a dimer, it binds to a glucocorticoid recognition sequence (GRE) on the 5'-upstream promoter sequence of glucocorticoid-responsive genes. GREs may increase transcription and negative (n)GREs may decrease transcription, resulting in increased or decreased mRNA and protein synthesis.

preventing the unoccupied GR localizing to the nuclear compartment. Once the glucocorticoid binds to GR, hsp90 dissociates, thus exposing two nuclear localization signals and allowing the activated GR–glucocorticoid complex to rapidly move into the nucleus and bind to DNA (Figure 2).

EFFECTS ON GENE TRANSCRIPTION

Glucocorticoids produce their effect on responsive cells by activating GR to directly or indirectly regulate the transcription of certain target genes [6,7]. The number of genes per cell directly regulated by glucocorticoids is estimated to be between 10 and 100, but many genes are indirectly regulated through an interaction with other transcription factors, as discussed below. Upon activation GR forms a homodimer which binds to DNA at consensus sites termed glucocorticoid response elements (GRE) in the 5'-upstream promoter region of glucocorticoid-responsive genes. The way in which activated GR seeks out the small number of GREs in approximately 100000 genes is not completely understood, but recent evidence suggests that the GR dimer binds non-specifically to DNA then attaches to another strand of DNA before dissociating from the first site of attachment. This is repeated until a high-affinity GRE site is encountered, the GR thus moving through the genome like Tarzan swinging through the jungle [8]. The binding of the GR dimer to GRE changes the rate of transcription, resulting in either induction or repression of the gene. The consensus sequence for GRE binding is the palindromic 15-bp sequence GGTACAnnnTGTTCT (where n is any nucleotide), although for repression of transcription the putative negative GRE has a more variable sequence. Negative GREs have only rarely been identified in some genes, such as the pro-opiomelanocortin gene [9]. The repression of the osteocalcin gene

by glucocorticoids appears to be due to binding of the GR dimer to a GRE which overlaps the TATA box and therefore interferes with the initiation of transcription [10]. Most genes that are repressed by glucocorticoids have no GRE, however, suggesting that some other mechanism must be invoked.

Crystallographic studies indicate that the zinc finger binding to DNA occurs within the major groove of DNA with one finger from each component of the dimer interacting with one half of the palindrome [11]. In contrast to these simple GREs, there are 'composite' GREs that do not share these GRE sequences, but depend on the presence of other transcription factors binding to DNA [12]. GR may also bind to less well-defined regions of DNA and regulate promoters that contain no obvious GRE sequences. Interaction with other transcription factors may also be important in determining differential glucocorticoid responsiveness in different cell types. Other transcription factors binding in the vicinity of GRE may have a powerful influence on glucocorticoid inducibility and the relative abundance of different transcription factors may contribute to the glucocorticoid responsiveness of a particular cell type.

GR may also inhibit protein synthesis by reducing the stability of mRNA via enhanced transcription of specific ribonucleases that break down mRNA containing constitutive AU-rich sequences in the untranslated 3'-region, thus shortening the turnover time of mRNA. This is a mechanism whereby glucocorticoids inhibit the synthesis of the cytokine granulocyte–macrophage colony-stimulating factor (GM-CSF), which plays a key role in the survival of inflammatory cells at the site of inflammation [13]. Such a mechanism may also be important for the repressive effect of glucocorticoids on inducible cyclo-oxygenase (COX-2) [14]. This may be an important mechanism whereby glucocorticoids inhibit some inflammatory genes.

INTERACTION WITH TRANSCRIPTION FACTORS

GR may interact directly with other transcription factors, which bind to each other via so-called leucine zipper interactions [15,16]. This could be an important determinant of glucocorticoid responsiveness and is a key mechanism whereby glucocorticoids exert their anti-inflammatory actions [17]. This interaction was first demonstrated for the collagenase gene which is induced by the transcription factor activator protein-1 (AP-1), a heterodimer of Fos and Jun oncoproteins. AP-1 binds to specific DNA binding sites (TRE or TPA response element, TGACTCA). Glucocorticoids are potent inhibitors of collagenase gene transcription induced by tumour necrosis factor-α (TNF-α) and phorbol esters, which both activate AP-1. AP-1 forms a protein–protein complex with activated GR, and this prevents GR interacting with DNA and thereby reduces glucocorticoid responsiveness. In human lung TNF-α and phorbol esters increase AP-1 binding to DNA and this is inhibited by glucocorticoids [18,19].

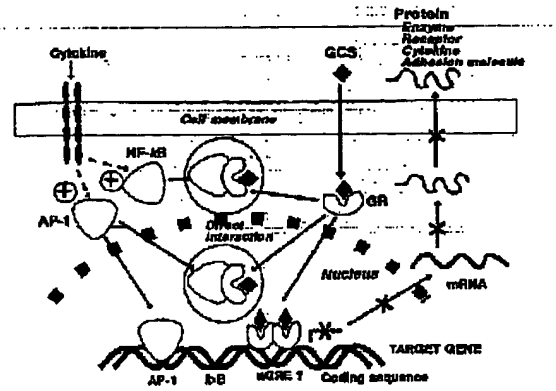

Figure 3 Direct interaction between the transcription factors AP-1 and NF-κB and the GR may result in mutual repression

In this way glucocorticoids may counteract the chronic inflammatory effects of cytokines which activate these transcription factors.

AP-1 may be important in regulating the expression of inflammatory genes in concert with other transcription factors, such as nuclear factor-κB (NF-κB).

NF-κB plays a critical part in regulating the expression of many inflammatory and immune genes and may play an amplifying role in the inflammatory process [20]. GR may interact with NF-κB in a similar manner by a direct protein–protein interaction, thus inhibiting the expression of a wide range of inflammatory genes [18,19,21–23] (Figure 3).

β_2-Adrenergic agonists, via cyclic AMP formation and activation of protein kinase A, result in the activation of the transcription factor CREB which binds to a cyclic AMP responsive element (CRE) on genes. A direct interaction between CREB and GR has been demonstrated [24]. β-Agonists increase CRE binding in human lung and epithelial cells *in vitro* and at the same time reduce GRE binding, suggesting that there may be a protein–protein interaction between CREB and GR within the nucleus [25]. However, in some cell lines cyclic AMP increases the transcriptional effects of glucocorticoids [26].

The interaction of GR with another family of transcription factors, signal transducers and activators of transcription (STATs), which are important for the signalling of many cytokines, has also been demonstrated. A positive interaction between GR and STAT5 and STAT6 has been shown, suggesting that glucocorticoids may enhance the effects of certain cytokines [27].

These interactions between activated GR and transcription factors occur within the nucleus, but protein–protein interactions may also occur in the cytoplasm [28].

EFFECTS ON CHROMATIN STRUCTURE

There has recently been increasing evidence that glucocorticoids may have effects on the chromatin structure. DNA in chromosomes is wound around

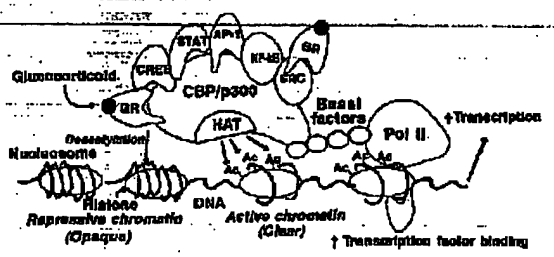

Figure 4 Effect of glucocorticoids on chromatin structure

Transcription factors such as STATs, AP-1 and NF-κB bind to co-activator molecules, such as CREB binding protein (CBP) or p300, which have intrinsic histone acetyltransferase (HAT) activity, resulting in acetylation (Ac) of histone residues. This leads to unwinding of DNA and allows increased binding of transcription factors resulting in increased gene transcription. Glucocorticoid receptors (GR) after activation by glucocorticoids may bind to a glucocorticoid receptor co-activator (SRC) which is bound to CBP and results in increased transcription. Activated GR, probably through binding to a co-repressor molecule, may also deacetylate histone, with increased coiling of DNA around histone, thus preventing transcription factor binding leading to gene repression.

histone molecules in the form of nucleosomes. Several transcription factors interact with large co-activator molecules, such as CREB binding protein (CBP) and the related p300, which bind to the basal transcription factor apparatus [29]. Several transcription factors have now been shown to bind directly to CBP, including AP-1, NF-κB and STATs [30–33]. Since binding sites on this molecule may be limited, this may result in competition between transcription factors for the limited binding sites available, so that there is an indirect rather than a direct protein–protein interaction (Figure 4). CBP also interacts with nuclear hormone receptors such as GR. These nuclear hormone receptors may interact with CBP and the basal transcriptional apparatus through binding to other nuclear co-activator proteins, including glucocorticoid receptor co-activator-1 (SRC-1) [34,35], transcription factor intermediary factor-2 (TIF-2) or glucocorticoid receptor interacting protein-1 (GRIP-1) [36]. A newly described nuclear protein called p300/CBP co-integrator-associated protein (p/CIP) appears to be particularly important in the binding of several nuclear receptors to CBP/p300 [37]. These nuclear activator proteins associate with nuclear receptors via a common sequence, LXXLL (where L is lysine and X is any amino acid) [38].

DNA is wound around histone proteins to form nucleosomes and the chromatin fibre in chromosomes. At a microscopic level that chromatin may become dense or opaque due to the winding or unwinding of DNA around the histone core. CBP and p300 have histone acetylation activity which is activated by the binding of transcription factors such as AP-1 and NF-κB [39]. Acetylation of histone residues results in unwinding of DNA coiled around the histone core, thus opening up the chromatin structure, which allows transcription factors to bind more readily, thereby increasing transcription (Figure 4). Repression of genes reverses this process by histone deacetylation [40]. The process of deacetylation involves the binding of hormone or vitamin receptors to co-repressor molecules such as nuclear receptor co-repressor (N-CoR), which forms a complex with another repressor molecule, Sin3, and a histone deacetylase [41,42]. Deacetylation of histone increases the winding of DNA round histone residues, resulting in dense chromatin structure and reduced access of transcription factors to their binding sites, thereby leading to repressed transcription of inflammatory genes. Activated GR may bind to several transcription co-repressor molecules that associate with proteins with histone deacetylase activity, with consequent repression of inflammatory genes by the mechanism described [40] (Figure 4).

TARGET GENES IN INFLAMMATION CONTROL

Glucocorticoids may control inflammation by inhibiting many aspects of the inflammatory process through increasing the transcription of anti-inflammatory genes and decreasing the transcription of inflammatory genes [2,17] (Table 1).

Anti-inflammatory proteins

Glucocorticoids may suppress inflammation by increasing the synthesis of several anti-inflammatory proteins. Glucocorticoids increase the synthesis of lipocortin-1, a 37 kDa protein that has an inhibitory effect on phospholipase A_2 (PLA_2), and therefore may inhibit the production of lipid mediators. Glucocorticoids induce the formation of lipocortin-1 in several cells and recombinant lipocortin-1 has acute anti-inflammatory properties [43]. However, glucocorticoids do not induce lipocortin-1 expression in many cell types and, indeed, the inhibitory effect of lipocortin on PLA_2 is largely an artefact and due to depletion of membrane phospholipids [44].

Glucocorticoids also increase the synthesis of se-

Table 1 Effect of glucocorticoids on gene transcription

Increased transcription
Lipocortin-1
β_2-Adrenoceptor
Secretory leucocyte inhibitory protein
Clara cell protein (CC10)
IL-1 receptor antagonist
IL-1R2 (decoy receptor)
IκB-α
Decreased transcription
Cytokines
(IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-11, IL-12, IL-13, TNF-α, GM-CSF, stem cell factor)
Chemokines
(IL-8, RANTES, MIP-1α, MCP-1, MCP-3, MCP-4, eotaxin)
iNOS
COX-2
Cytoplasmic PLA_2
Endothelin-1
NK_1-receptors, NK_2-receptors
Adhesion molecules (ICAM-1, E-selectin)

cretory leucocyte protease inhibitor in human airway epithelial cells by increasing gene transcription [45]. Secretory leucocyte protease inhibitor is the predominant antiprotease in airways and may be important in reducing airway inflammation by counteracting inflammatory enzymes such as tryptase. Clara cell protein (CC10), a 10 kDa protein secreted by epithelial cells which has anti-inflammatory and immunomodulatory properties, is also increased by glucocorticoids [46].

Interleukin (IL)-1 receptor antagonist is a cytokine that blocks the binding of IL-1 to its receptors. Its synthesis is increased by glucocorticoids, thus counteracting the effect of the pro-inflammatory cytokine IL-1. Therefore, treatment of asthmatic patients with inhaled glucocorticoids results in an increased expression of IL-1 receptor antagonist in airway epithelial cells *in vitro* and *in vivo* [47,48]. IL-1 interacts with two types of surface receptor, designated IL-1R1 and IL-1R2. The inflammatory effects of IL-1β are mediated exclusively via IL-1R1, whereas IL-1R2 has no signalling activity, but binds IL-1 and therefore acts as a 'molecular decoy' that interferes with the actions of IL-1. Glucocorticoids are potent inducers of this decoy IL-1 receptor and result in release of a soluble form of the receptor, thus reducing the functional activity of IL-1 [49].

IL-10 is an anti-inflammatory cytokine secreted predominantly by macrophages which inhibits the transcription of many pro-inflammatory cytokines, chemokines and inflammatory enzymes, and this appears to be mediated, at least in part, via an inhibitory effect on NF-κB [50]. IL-10 secretion by alveolar macrophages may be impaired in patients with asthma, resulting in increased macrophage cytokine secretion [51]. Glucocorticoid treatment in patients with asthma increases IL-10 secretion by these cells, although this appears to be an indirect effect, since treatment of alveolar macrophages *in vitro* with glucocorticoids tends to decrease IL-10 secretion [51].

NF-κB is regulated by the inhibitory protein IκB to which it is bound in the cytoplasm [20]. There is some evidence that glucocorticoids increase the synthesis and transcription of the predominant form of IκB, IκB-α, in mononuclear cells and T-lymphocytes, thus terminating the activation of NF-κB [52,53], but this has not been seen in other cell types [54–56]. The IκB-α gene does not appear to have any GRE consensus sequence, so any effect of glucocorticoids is probably mediated via other transcription factors.

In epithelial cells glucocorticoids also increase the expression of the enzyme neutral endopeptidase, which degrades inflammatory peptides such as substance P, bradykinin and endothelin-1 [57]. Patients with asthma treated with inhaled glucocorticoids have a higher level of neutral endopeptidase expression than untreated patients [58].

β_2-Adrenoceptors

Glucocorticoids increase the expression of β_2-adrenoceptors by increasing the rate of transcription and the human β_2-receptor gene has three potential GREs [59]. Glucocorticoids double the rate of β_2-receptor gene transcription in human lung *in vitro*, resulting in increased expression of β_2-receptors [60]. Using autoradiographic mapping and *in situ* hybridization in animals to localize the increase in β_2-receptor expression, there appears to be an increase in all cell types, including airway epithelial cells and airway smooth muscle, after chronic glucocorticoid treatment [61]. This may be relevant in asthma as it may prevent down-regulation in response to prolonged treatment with β_2-agonists. In rats glucocorticoids prevent the down-regulation and reduced transcription of β_2-receptors in response to chronic β-agonist exposure [61], although inhaled glucocorticoids have not been shown to prevent tolerance to the bronchoprotective effects of an inhaled β_2-agonist [62].

Cytokines

Although it is not yet possible to be certain of the most critical aspects of glucocorticoid action in chronic inflammatory diseases such as asthma, it is likely that their inhibitory effects on cytokine synthesis are of particular importance. Glucocorticoids inhibit the transcription of several cytokines that are relevant in inflammatory diseases, including IL-1β, IL-2, IL-3, IL-6, IL-11, TNF-α, GM-CSF and chemokines that attract inflammatory cells to the site of inflammation, including IL-8, RANTES, MCP-1, MCP-3, MCP-4, MIP-1α and eotaxin. In allergic inflammation the expression of cytokines IL-4 (critical for IgE synthesis) and IL-5 (critical for eosinophilic inflammation) is also inhibited by glucocorticoids. These inhibitory effects were at one time thought to be mediated directly via interaction of GR with a negative GRE in the upstream promoter sequence of the cytokine gene, resulting in gene repression. However, there is no negative GRE consensus sequence in the upstream promoter region of any of these cytokine genes, suggesting that glucocorticoids inhibit transcription indirectly. For example, the 5′-promoter sequence of the human IL-2 gene has no GRE consensus sequences, yet glucocorticoids are potent inhibitors of IL-2 gene transcription in T-lymphocytes. Transcription of the IL-2 gene is predominantly regulated by a cell-specific transcription factor, nuclear factor of activated T-cells (NF-AT), which is activated in the cytoplasm on T-cell receptor stimulation via calcineurin. A nuclear factor is also necessary for increased activation and this factor has been identified as AP-1, which binds directly to NF-AT to form a transcriptional complex [63]. Glucocorticoids therefore inhibit IL-2 gene transcription indirectly by binding to AP-1, thus preventing increased transcription due to NF-AT [64]. Inhibition of IL-5 gene transcription may involve a similar mechanism [65]. Another example of a cytokine gene negatively regulated by glucocorticoids that does not have a GRE in its promoter region is RANTES, which is regulated predominantly by NF-κB and AP-1 [66]. Glucocorticoids therefore appear to inhibit

cytokine gene expression by inhibiting transcription factors that regulate their expression, rather than by binding to their promoter regions.

There may be marked differences in the response of different cells and of different cytokines to the inhibitory action of glucocorticoids and this may be dependent on the relative abundance of transcription factors. Thus in alveolar macrophages and peripheral blood monocytes GM-CSF secretion is more potently inhibited by glucocorticoids than IL-1β or IL-6 secretion [67]. This might be explained by the need for different combinations of transcription factor activation for optimal gene transcription, so that glucocorticoid sensitivity may be determined by the particular combination of transcription factors needed and their propensity for activation in different cell types.

Inflammatory enzymes

Nitric oxide (NO) synthase is inducible by pro-inflammatory cytokines, resulting in increased NO production. NO may increase blood flow and plasma exudation and may amplify the inflammatory response. In the airways NO may contribute to the plasma exudation seen in asthma and other inflammatory diseases, and may amplify eosinophilic inflammation in asthma by tipping the immune balance in favour of Th2 lymphocytes that secrete IL-4 and IL-5, by acting as a chemotactic agent for eosinophils and by increasing eosinophil survival [68,69]. The induction of the inducible form of NO synthase (iNOS) is potently inhibited by glucocorticoids [70]. In cultured human pulmonary epithelial cells pro-inflammatory cytokines result in increased expression of iNOS and increased NO formation due to increased transcription of the iNOS gene and this is inhibited by glucocorticoids [71]. There is no negative GRE in the promoter sequence of the iNOS gene, but NF-κB appears to be the most important transcription factor in regulating iNOS gene transcription [72]. Since TNF-α, IL-1β and oxidants activate NF-κB in airway epithelial cells, this accounts for their activation of iNOS expression. Glucocorticoids may therefore prevent induction of iNOS by inhibiting NF-κB, thereby inhibiting transcription. The increased expression of iNOS in the airways of patients with asthma results in an increase in the level of NO in the exhaled air [73] and this is inhibited by inhaled glucocorticoids [74].

Glucocorticoids inhibit the synthesis of several inflammatory mediators implicated in inflammation through an inhibitory effect on enzyme induction. Glucocorticoids inhibit the induction of the gene coding for COX-2 in monocytes and epithelial cells and this also appears to be via NF-κB activation [75–77] and by a post-transcriptional effect on mRNA stability [14]. Glucocorticoids also inhibit the gene transcription of a form of phospholipase A_2 (cPLA$_2$) induced by cytokines [77]. However, glucocorticoids do not appear to modulate expression of the 5'-lipoxygenase and studies of cysteinyl-leukotriene formation in patients with asthma *in-vivo* indicate that doses of oral or inhaled glucocorticoids that are effective clinically do not significantly reduce the excretion of leukotriene E_4, the major stable metabolite of leukotriene D_4 [78].

Glucocorticoids also inhibit the synthesis of endothelin-1 in lung [79] and airway epithelial cells and this effect may also be via inhibition of transcription factors that regulate its expression [80].

Inflammatory receptors

Glucocorticoids also decrease the transcription of genes coding for certain receptors that are involved in the inflammatory process. Thus the NK_1-receptor, which mediates the inflammatory effects of tachykinins in the airways, has increased gene expression in asthma [81]. This may be inhibited by glucocorticoids through an interaction with AP-1, as the NK_1 receptor gene promoter region has no GRE, but has an AP-1 response element [82]. Similarly NK_2-receptor expression is also reduced by glucocorticoids [83].

Cell survival

Glucocorticoids markedly reduce the survival of certain inflammatory cells such as eosinophils and T-lymphocytes. Eosinophil survival is dependent on the presence of cytokines such as IL-5 and GM-CSF. Exposure to glucocorticoids blocks the effects of these cytokines and leads to programmed cell death or apoptosis [84]. Glucocorticoids also promote apoptosis of T-lymphocytes. The molecular mechanism of action of glucocorticoids in increasing apoptosis in eosinophils and T-lymphocytes is uncertain and there are many potential sites of action, including effects on endogenous inhibitors of the apoptotic pathway. In contrast, glucocorticoids decrease apoptosis and therefore increase the survival of neutrophils [85,86]. The molecular mechanisms that account for the opposing effects of glucocorticoids on these two types of granulocyte are not yet certain.

Adhesion molecules

Adhesion molecules play a key role in the trafficking of inflammatory cells to sites of inflammation. The expression of many adhesion molecules on endothelial cells is induced by cytokines and glucocorticoids may lead indirectly to a reduced expression via their inhibitory effects on cytokines such as IL-1β and TNF-α. Glucocorticoids may also have a direct inhibitory effect on the expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and E-selectin at the level of gene transcription [87]. ICAM-1 expression in bronchial epithelial cell lines and monocytes is inhibited by glucocorticoids [88].

EFFECTS ON CELL FUNCTION

Glucocorticoids may have direct inhibitory actions on many inflammatory and structural cells involved in inflammation.

Macrophages

Glucocorticoids inhibit the release of inflammatory mediators and cytokines from alveolar macrophages *in vitro* [67,89], although their effect after inhalation *in vivo* is modest [90]. Glucocorticoids may be more effective in inhibiting cytokine release from alveolar macrophages than in inhibition of lipid mediators and reactive oxygen species *in vitro* [91,92]. Inhaled glucocorticoids reduce the secretion of chemokines and pro-inflammatory cytokines from alveolar macrophages in patients with asthma, whereas the secretion of IL-10 is increased [51]. Oral prednisone inhibits the increased gene expression of IL-1β in alveolar macrophages obtained by bronchoalveolar lavage from patients with asthma [93].

Eosinophils

Glucocorticoids have a direct inhibitory effect on mediator release from eosinophils, although they are only weakly effective in inhibiting secretion of reactive oxygen species and eosinophil basic proteins [94,95]. Glucocorticoids inhibit the permissive action of GM-CSF and IL-5 on eosinophil survival [96,97]. The increased apoptosis contributes to the reduction in airway eosinophils seen with glucocorticoid therapy. One of the best described actions of glucocorticoids in asthma is a reduction in circulating eosinophils, which may reflect an action on eosinophil production in the bone marrow. In patients with asthma there is an increase in the proportion of low-density eosinophils in the circulation, which may reflect an effect of cytokines [98]. Inhaled glucocorticoids inhibit the increase in circulating eosinophil count at night in patients with nocturnal asthma and also reduce plasma concentrations of eosinophil cationic protein [99]. After inhaled glucocorticoids there is a marked reduction in the number of low-density eosinophils, presumably reflecting inhibition of cytokine production in the airways [100].

T-lymphocytes

T-lymphocytes play a key role in orchestrating chronic inflammation and glucocorticoids are very effective in inhibiting activation, proliferation and survival of these cells, and in blocking the release of lymphokines such as IL-2, IL-3, IL-4, IL-5, IL-13 and GM-CSF, which are likely to play an important role in the recruitment and survival of inflammatory cells.

Mast cells

While glucocorticoids do not appear to have a direct inhibitory effect on mediator release from mast cells [101], chronic inhaled glucocorticoid treatment is associated with a marked reduction in mucosal mast cell number in airways [102]. This may be linked to a reduction in IL-3 and stem cell factor production, which is necessary for mast cell expression at mucosal surfaces. Mast cells also secrete various cytokines (TNF-α, IL-4, IL-5, IL-6, IL-8), but whether this is inhibited by glucocorticoids is not yet certain.

Dendritic cells

Dendritic cells in the epithelium of the respiratory tract appear to play a critical role in antigen presentation in the lung as they have the capacity to take up allergen, process it into peptides and present it via MHC molecules on the cell surface to uncommitted T-lymphocytes [103]. In experimental animals the number of dendritic cells is markedly reduced by systemic and inhaled glucocorticoids, thus dampening the immune response in the airways [104]. Topical glucocorticoids markedly reduce the number of dendritic cells in the nasal mucosa [105], and it is likely that a similar effect would be seen in airways.

Neutrophils

Neutrophils, which are not prominent in the biopsies of patients with asthma, are not very sensitive to the effects of glucocorticoids. Indeed systemic glucocorticoids increase peripheral neutrophil counts, which may reflect the increased survival time due to an inhibitory action of neutrophil apoptosis [85,86].

Endothelial cells

GR gene expression in the airways is most prominent in endothelial cells of the bronchial circulation and airway epithelial cells [106]. Glucocorticoids do not appear to directly inhibit the expression of adhesion molecules, although they may inhibit cell adhesion indirectly by suppression of cytokines involved in the regulation of adhesion molecule expression. Glucocorticoids may have an inhibitory action on airway microvascular leak induced by inflammatory mediators [107,108]. This appears to be a direct effect on postcapillary venular epithelial cells. The mechanism for this antipermeability effect has not been fully elucidated, but there is evidence that synthesis of a 100 kDa protein distinct from lipocortin-1 termed vasocortin may be involved [109]. Although there have been no direct measurements of the effects of glucocorticoids on airway microvascular leakage in asthmatic airways, regular treatment with inhaled glucocorticoids decreases the elevated plasma proteins found in bronchoalveolar lavage fluid of patients with stable asthma [110].

Epithelial cells

Epithelial cells may be an important source of inflammatory mediators in asthmatic airways and may drive and amplify the inflammatory response in the airways [111,112]. Airway epithelium may be one of the most important targets for inhaled glucocorticoids in asthma [3,113]. Glucocorticoids inhibit the increased transcription of the IL-8 gene induced by TNF-α in cultured human airway epithelial cells *in*

vitro [114,115] and the transcription of the RANTES gene in epithelial cells [116,117]. Inhaled glucocorticoids inhibit the increased expression of GM-CSF and RANTES in the epithelium of patients with asthma [111,118,119].

Glucocorticoids decrease the transcription of other inflammatory proteins in airway epithelial cells, including iNOS, COX-2, $cPLA_2$ and endothelin-1 [71,75,80]. Airway epithelial cells may be the key cellular target of inhaled glucocorticoids; by inhibiting the transcription of several inflammatory genes inhaled glucocorticoids may reduce inflammation in the airway wall.

Mucus secretion

Glucocorticoids inhibit mucus secretion in airways and this may be by a direct action on submucosal gland cells [120]. Recent studies suggest that glucocorticoids may also inhibit the expression of mucin genes such as *MUC2* and *MUC5A* [121]. In addition there are indirect inhibitory effects due to the reduction in inflammatory mediators that stimulate increased mucus secretion.

Neurogenic inflammation

Neurogenic inflammation, due to release of neuropeptides such as tachykinins from sensory nerves, may amplify inflammatory responses. Glucocorticoids may inhibit several aspects of neurogenic inflammation, including the synthesis of tachykinins by repression of the preprotachykinin-A gene [122], reduced expression of tachykinin receptors [81,83] and by increasing expression of neutral endopeptidase which degrades tachykinins [57].

GLUCOCORTICOID RESISTANCE

Although glucocorticoids are highly effective in the control of chronic inflammatory or immune diseases, a small proportion of patients will fail to respond even to high doses of oral glucocorticoids. This has been most extensively studied in asthma, as it is easier to assess the clinical response to glucocorticoids in this condition [123–125], although resistance to the therapeutic effects of glucocorticoids is also recognized in other inflammatory diseases, including rheumatoid arthritis and inflammatory bowel disease [126]. Glucocorticoid-resistant patients, although uncommon, present considerable management problems. Recognition of patients with glucocorticoid resistance is important, as elucidation of the molecular mechanisms may contribute to our understanding of glucocorticoid action and inflammatory disease mechanisms.

Clinical features of glucocorticoid-resistant asthma

Glucocorticoid resistance in asthma was first described by Schwartz et al. [127] in 1968 in six patients with asthma who did not respond clinically to high doses of systemic glucocorticoids and in whom there was also a reduced eosinopenic response. Larger groups of patients with chronic asthma who were glucocorticoid resistant were subsequently identified [128]. These patients failed to improve their mean peak expiratory flow by > 15% after taking prednisolone, 20 mg daily for at least 7 days. These patients are not Addisonian and do not suffer from the abnormalities in sex hormones described in familial glucocorticoid resistance (see below). Plasma cortisol and adrenal suppression in response to exogenous cortisol is normal [129]. Complete glucocorticoid resistance in asthma is very rare, but reduced responsiveness is more common, so that oral glucocorticoids are needed to control asthma adequately (steroid-dependent asthma).

Glucocorticoid-resistant (GCR) asthma is defined by a failure to improve lung function by > 15% after treatment with 30 to 40 mg of prednisolone for 2 weeks. These patients show the typical diurnal variability in peak expiratory flow and bronchodilate with inhaled β_2-agonists. Fibre-optic biopsies in patients with GCR asthma show the typical eosinophilic inflammation observed in patients with glucocorticoid-sensitive (GCS) asthma [130].

Mechanisms of glucocorticoid resistance

There may be several mechanisms for resistance to the effects of glucocorticoids. Although a family history of asthma is more common in patients with GCR than GCS asthma, little is known about its inheritance. It is possible that a certain proportion of the population has glucocorticoid resistance which only becomes manifest when they develop a severe immunological or immune disease that requires glucocorticoid therapy. Resistance to the inflammatory and immune effects of glucocorticoids should be distinguished from the rare familial glucocorticoid resistance, where there is an abnormality of glucocorticoid binding to GR.

Familial glucocorticoid resistance. Familial glucocorticoid resistance (FGR) is an extremely rare syndrome characterized by high circulating levels of cortisol without signs or symptoms of Cushing's syndrome [131]. Clinical manifestations, which may be absent, are due to an excess of non-glucocorticoid adrenal steroids, stimulated by high adrenocorticotropic hormone levels, resulting in hypertension with hypokalaemia and/or signs of androgen excess (usually hirsutism and menstrual abnormalities in females). Inheritance may be recessive, but only about 12 cases have so far been reported. Several abnormalities in GR function have been described in peripheral blood leucocytes or fibroblasts from these patients. These include a decreased affinity of GR for cortisol, a reduced number of GRs, GR thermolability and an abnormality in the binding of the GR complex to DNA. The molecular basis of the disease in patients with a reduction in GR appears to be a point mutation

in the glucocorticoid binding domain of GR [132]. Glucocorticoid resistance may occur in certain malignancies and this may be due to abnormal expression of GR. Thus, in multiple myeloma, resistance to the inhibitory effect of glucocorticoids is associated with the expression of a truncated form of GR mRNA with reduced stability, resulting in a shortened form of GR with markedly reduced glucocorticoid binding [133].

Resistance to anti-inflammatory actions of glucocorticoids. Resistance to the anti-inflammatory and immunomodulatory effects of glucocorticoids differs from the FGR described above, as it is not associated with high circulating concentrations of cortisol or adrenocorticotropic hormone, and is not accompanied by hypertension, hypokalaemia or androgen excess [124]. Furthermore, these patients are not Addisonian and show normal adrenal suppression [129]. This suggests that any abnormality is unlikely to be due to those described for FGR in the glucocorticoid binding domain of GR. Indeed, chemical mutational analysis of GR has failed to demonstrate any major abnormality in predicted structure in GCR compared with GCS asthma [134]. Glucocorticoid resistance may be *primary* (inherited or acquired of unknown cause) or *secondary* to some factor that may reduce glucocorticoid responsiveness (glucocorticoids themselves, cytokines, β-adrenergic agonists). There are several possible mechanisms for a reduced anti-inflammatory response to glucocorticoids.

Pharmacokinetic abnormalities. The initial suggestion of Schwartz et al. [127] was that defective responses to glucocorticoids were due to increased clearance of the glucocorticoid, resulting in reduced clinical and eosinopenic response. There is no evidence for altered bioavailability or plasma clearance of prednisolone or methylprednisolone in patients with GCR asthma [135,136]. Metabolism of glucocorticoids may be increased by induction of cytochrome *P*-450 enzymes in response to certain drugs, which may thus lead to a secondary glucocorticoid resistance [137].

Antibodies to lipocortin-1. Some anti-inflammatory effects of glucocorticoids may be due to induction of lipocortin-1 [43]. In some patients with GCR rheumatoid arthritis, autoantibodies to lipocortin-1 have been described [138]. However, two independent studies have failed to demonstrate the presence of IgG or IgM lipocortin-1 antibodies in either GCR or steroid-dependent asthma [139,140].

Cellular abnormalities. Glucocorticoid resistance has been documented *in vitro*, with reduced suppression of activation antigens and cytokines in monocytes and T-lymphocytes from patients with GCR asthma [136,141–145]. There is no difference in the proportion of $CD4^+$ and $CD8^+$ T-lymphocytes in GCR patients, although there is increased expression of CD25 (IL-2 receptor) in GCR compared with GCS patients, indicating a greater degree of immune activation [145]. These studies in circulating leucocytes suggest that the defect in glucocorticoid responsiveness extends outside the respiratory tract and is therefore unlikely to be explained entirely by inflammatory changes in the airways. In patients with GCR asthma a reduced blanching response to topical glucocorticoids applied to the skin further indicates that there is a generalized abnormality that is unlikely to be secondary to local cytokine production [146]. In patients with GCS asthma there was suppression of the cutaneous tuberculin response after treatment for 2 weeks with oral prednisolone associated with reduced numbers of macrophages, eosinophils and activated T-lymphocytes, but this was not observed in GCR patients [147].

Abnormality in GR function. In FGR there is a structural abnormality in GR that results in reduced glucocorticoid binding affinity. In GCR asthma either no difference in GR affinity and receptor density or a relatively small reduction in GR affinity has been reported [135,145,148,149]. Two types of glucocorticoid resistance have been described: a reduced affinity of GR binding confined to T-lymphocytes which reverts to normal after 48 h in culture, and a much less common reduction in GR density (in only 2/17 GCR patients), which does not normalize with prolonged incubation [149]. This suggests that there may be different types of GCR asthma. The small reduction in GR affinity is unlikely to be of functional significance and is not associated with elevated plasma cortisol, unlike patients with FGR. The small reduction in GR affinity may be secondary to cytokine exposure, since the normalization of GR affinity *in vitro* is prevented by a combination of IL-2 and IL-4 [149] and this combination of cytokines reduces the binding affinity in nuclear GR in T-lymphocytes, although either cytokine alone has no effect [150]. The IL-4-like cytokine IL-13 has a similar effect and is effective alone [151]. This suggests that glucocorticoid resistance may occur in the airways of patients with asthma as a secondary phenomenon due to the local production of cytokines. In patients with GCR asthma there is a significant increase in the numbers of bronchoalveolar lavage cells expressing IL-2 and IL-4 mRNA compared with patients with GCS asthma, but no difference in interferon-γ mRNA-positive cells. After oral prednisone for 1 week there is a reduction in IL-4-expressing cells and a rise in interferon-γ-positive cells in GCS asthma, whereas in GCR asthma there is no fall in IL-4-positive cells and a fall in interferon-γ-positive cells [130]. This may indicate that there are different patterns of cytokine release which may contribute to glucocorticoid resistance. Although this may account for the increased requirement for glucocorticoids in more severe asthma, it is unlikely to explain the reduced glucocorticoid response seen in circulating mononuclear cells and in the skin of patients with no response to oral glucocorticoids.

The recognition that there is a splice variant of GR, GR-β, that does not bind glucocorticoids, but binds to GRE [4], has suggested that this might be a mechanism of glucocorticoid resistance if GR-β is produced in excess. There is some evidence for an increase in GR-β is induced by inflammatory cytokines and there is an increase in GR-β-producing cells in GCR patients [152]. However, it appears to be unlikely that GR-β

abnormality in the interaction between GR and AP-1 [155]. This defect does not appear to apply to the other transcription factors, NF-κB and CREB, that also interact with GR [155]. The abnormality in the interaction between GR and AP-1 is unlikely to be due to a defect in GR, since the protein sequence of GR in patients with GCR asthma appears to be normal [134]. It is more likely to be due to a defect in AP-1 or its activation. Indeed, activation of c-Fos by phorbol esters is potentiated in the cells of patients with GCR compared with GCS asthma [156], and preliminary evidence suggests that one of the key enzymes involved in activation of AP-1, namely Jun N-terminal kinase (JNK) is abnormally activated in these patients [157]. The increased basal and cytokine-induced AP-1 activity may lead to consumption of GR, so that glucocorticoids are not able to suppress the inflammatory response, either through interacting with GRE or with other transcription factors such as NF-κB (Figure 5).

An abnormality in AP-1 may also account for the

Secondary glucocorticoid resistance

Although complete glucocorticoid resistance is uncommon, there may be a spectrum of glucocorticoid responsiveness in inflammatory diseases. This may reflect several mechanisms that are secondary either to disease activity itself or to the effects of therapy.

Down-regulation of GR. Down-regulation of GR in circulating lymphocytes after oral prednisolone has been demonstrated in normal individuals [158]. Whether high local concentrations of inhaled glucocorticoids reduce GR expression in surface cells of the airway, such as epithelial cells, is not yet certain, although patients with asthma treated with inhaled glucocorticoids do not appear to have a reduced expression of GR in the airways [106]. It is possible that certain individuals may be more susceptible to the effects of down-regulation. If effective GR density is reduced by direct interaction with other transcription factors, such as AP-1 and NF-κB, then the down-regulating effect of glucocorticoids on GR would be expected to have a greater functional consequence.

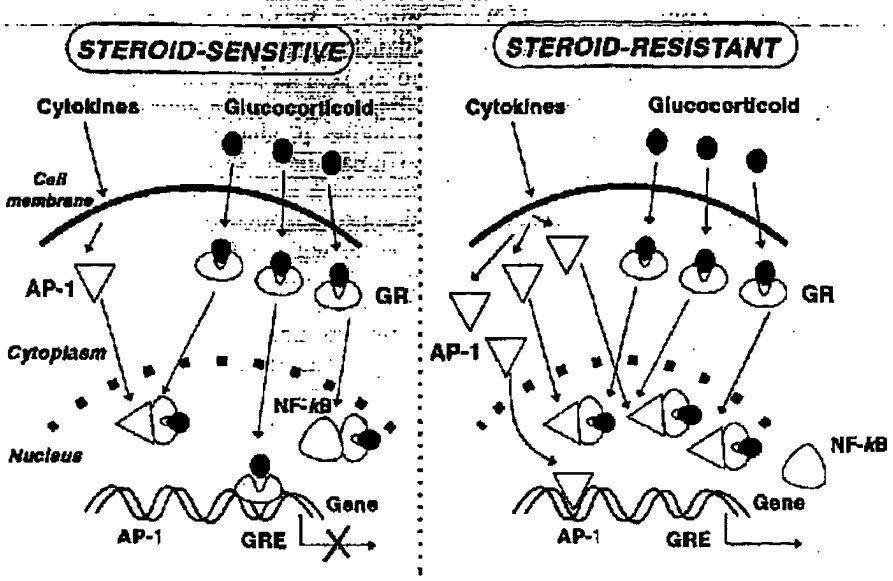

Figure 5 Proposed mechanism of primary glucocorticoid resistance in asthma

Increased activation of AP-1 results in the complexing of GRs, thus preventing the anti-inflammatory action of glucocorticoids, either through binding to GREs or through inhibition of NF-κB.

has any functional effect on GRE binding of GR-α [153].

There is a marked reduction in GR–GRE binding in the peripheral blood mononuclear cells of patients with GCR asthma and Scatchard analysis has demonstrated a marked reduction in GR available for DNA binding compared with cells from patients with GCS asthma [154].

Interaction between GR and transcription factors. In the peripheral blood mononuclear cells of patients with GCS asthma and normal control subjects the phorbol ester PMA, which activates AP-1, results in reduced GRE binding. This inhibitory effect is significantly reduced in the peripheral blood mononuclear cells of patients with GCR asthma, indicating an abnormality in the interaction between GR and AP-1 [155]. This defect does not appear to apply to the other transcription factors, NF-κB and CREB, that also interact with GR [155]. The abnormality in the interaction between GR and AP-1 is unlikely to be due to a defect in GR, since the protein sequence of GR in patients with GCR asthma appears to be normal [134]. It is more likely to be due to a defect in AP-1 or its activation. Indeed, activation of c-Fos by phorbol esters is potentiated in the cells of patients with GCR compared with GCS asthma [156], and preliminary evidence suggests that one of the key enzymes involved in activation of AP-1, namely Jun N-terminal kinase (JNK) is abnormally activated in these patients [157]. The increased basal and cytokine-induced AP-1 activity may lead to consumption of GR, so that glucocorticoids are not able to suppress the inflammatory response, either through interacting with GRE or with other transcription factors such as NF-κB (Figure 5).

An abnormality in AP-1 may also account for the selective resistance to the effects of glucocorticoid in GCR asthma, since AP-1 is more likely to be important in the regulation of some genes than in others. It would also explain why resistance is seen to the anti-inflammatory effects but not to the endocrine or metabolic effects of glucocorticoids, since such resistance can only arise when AP-1 is activated at the inflammatory site, whereas the hormonal effects of glucocorticoids at uninflamed sites will not be impaired. Furthermore, there may also be differences in the glucocorticoid resistance of different target cells, depending upon the relative balance of transcription factors.

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Effects of cytokines. Several pro-inflammatory cytokines, including IL-1β, IL-6 and TNF-α, activate AP-1 and NF-κB in human lung [18,159]. As all these cytokines are secreted in asthmatic inflammation, this suggests that these transcription factors will be activated in the cells of asthmatic airways. These activated transcription factors may then form protein–protein complexes with activated GR, both in the cytoplasm and within the nucleus, thus reducing the number of effective GRs and thereby decreasing glucocorticoid responsiveness [17]. In a model *in vitro* system increased expression of c-Fos or c-Jun oncoproteins prevents the activation of mouse mammary tumour virus promoter by GR, thus creating a model of glucocorticoid resistance [160]. Addition of recombinant c-Jun or c-Fos proteins to partially purified GR results in inhibition of DNA binding [160]. Phorbol esters, which activate AP-1, result in attenuation of glucocorticoid-mediated gene activation [161]. Any reduction in glucocorticoid responsiveness would be greater as the intensity of asthmatic inflammation increased and may contribute, for example, to the failure of oral or intravenous glucocorticoids to control acute exacerbations of asthma. Once the inflammation is brought under control with large doses of oral glucocorticoids, glucocorticoid responsiveness increases again so that lower doses of inhaled or oral glucocorticoids are needed to control the inflammation.

Increased resistance may also be due to the effects of cytokines on GR function, since high concentrations of IL-2 and IL-4 have been shown to reduce GR affinity in T-lymphocytes *in vitro* [150]. This effect would only be seen in mucosal T-lymphocytes of patients with severe asthma and it is therefore difficult to obtain evidence to support this possibility.

Effect of β_2-agonists. High concentrations of β_2-agonists activate CREB in rat and human lung and in inflammatory cells via an increase in cyclic AMP concentration [19,25]. This results in reduced GRE binding due to the formation of GR–CREB complexes [25]. This predicts that high concentrations of β_2-agonists would induce glucocorticoid resistance. In patients with asthma, while 3 weeks of treatment with an inhaled glucocorticoid blocked the airway response to inhaled allergen, concomitant treatment with inhaled glucocorticoid and a relatively large dose of inhaled β-agonist appeared to provide no significant protection against allergen challenge [162]. This suggests that high doses of an inhaled β_2-agonist might interfere with the anti-asthma effect of inhaled glucocorticoids. It is possible that some patients who use very high doses of inhaled β_2-agonists (over two canisters per month of metered-dose inhalers or regular nebulized doses) may develop a degree of glucocorticoid resistance that is overcome by increasing the dose of inhaled or oral glucocorticoid. This may account for some of the deleterious effects of high-dose β-agonists on asthma mortality and morbidity [163]. The use of high doses of nebulized β_2-agonists in the treatment of acute exacerbations of asthma may result in resistance to the effects of high-dose intravenous glucocorticoids. Glucocorticoid responsiveness might be restored by reducing the dose of inhaled β_2-agonists.

THERAPEUTIC IMPLICATIONS

Greater understanding of the molecular mechanism whereby glucocorticoids suppress inflammation has opened up the potential for improvement in glucocorticoids and the development of novel anti-inflammatory drugs.

New glucocorticoids

The recognition that most of the anti-inflammatory effects of glucocorticoids are mediated by repression of transcription factors (transrepression), whereas the endocrine and metabolic effects of steroids are likely to be mediated via GRE binding (transactivation) has led to a search for novel corticosteroids that selectively transrepress, thus reducing the potential risk of systemic side effects. Since corticosteroids bind to the same GR, this seems at first to be an unlikely possibility, but while GRE binding involved a GR homodimer, interaction with transcription factors AP-1 and NF-κB involves only a single GR. A separation of transactivation and transrepression has been demonstrated using reporter gene constructs in transfected cells using selective mutations of GR [164]. Furthermore, some steroids, such as the antagonist RU486, have a greater transrepression than transactivation effect. Indeed, the topical steroids used in asthma therapy today, such as fluticasone propionate and budesonide, appear to have more potent transrepression than transactivation effects, which may account for their selection as potent anti-inflammatory agents [165]. Recently, a novel class of steroids has been described in which there is potent transrepression with relatively little transactivation. These 'dissociated' steroids, including RU24858 and RU40066, have anti-inflammatory effects *in vivo* [166]. This suggests that the development of steroids with a greater margin of safety is possible and may predict the development of oral steroids that are safe to use in inflammatory diseases.

NF-κB inhibitors

Since NF-κB appears to mediate many of the anti-inflammatory effects of glucocorticoids, this has led to a search for specific inhibitors of this transcription factor or its activating pathways [20,167]. Antioxidants have the ability to block activation of NF-κB in response to a wide variety of stimuli, and drugs such as pyrrolidine dithiocarbamate have proved useful for *in vitro* studies, but are too toxic for *in vivo* development [168]. Spin-trap antioxidants may be more effective since they work at an intracellular level

[169]. However, antioxidants do not block all of the effects of NF-κB and this may require the development of novel drugs.

Some naturally occurring NF-κB inhibitors have already been identified. Thus gliotoxin, derived from *Aspergillus*, is a potent NF-κB inhibitor which appears to be relatively specific [170]. The anti-inflammatory cytokine IL-10 also has an inhibitory effect on NF-κB, via an effect on IκB-α [50], and has been shown to be effective in management of chronic inflammatory diseases such as Crohn's disease, which is resistant to glucocorticoid therapy [171].

Novel approaches to inhibition of NF-κB would be to develop specific inhibitors of IκB kinases involved in the initial activation of NF-κB, to block the signal transduction pathways leading to activation of IκB kinases. Now that IκB kinases have been identified, it may be possible to screen and design specific inhibitors. It may also be possible to inhibit the activity of the enzymes responsible for its degradation of the IκB complex, although the proteasome has many other important functions and its inhibition is likely to produce severe side effects. Recently it has been possible to block NF-κB function by targeting of a specific enzyme (ubiquitin ligase) involved in conjugation of ubiquitin [172]. It may be more difficult to develop drugs to directly inhibit the components of NF-κB itself, but antisense oligonucleotides have been shown to be effective inhibitors *in vitro* and stable cell permeable phosphorothioate oligonucleotides are a therapeutic possibility in the future. Adenovirus-mediated gene transfer of IκB-α has been reported to inhibit endothelial cell activation [173].

It may be unwise, however, to block NF-κB for prolonged periods, as it plays such a critical role in immune and host defence responses. Targeted disruption ('knock-out') of p65 is lethal because of developmental abnormalities [174], whereas lack of p50 results in immune deficiencies and increased susceptibility to infection [175]. However, topical application of NF-κB inhibitors by inhalation may prove to be safe.

Drug interactions

There are complex interactions between transcription factors, either directly or via co-activator molecules such as CBP. This might be exploited therapeutically by a combination of drugs which act on different transcript factors or pathways that may work together co-operatively. For example, NF-AT has a cytoplasmic component (NF-ATp) which is blocked by cyclosporin and tacrolimus (FK506), and a nuclear component (AP-1) which is blocked by glucocorticoids. Combining steroids and cyclosporin may therefore have a synergistic inhibitory effect on the expression of genes such as IL-2, IL-4 and IL-5. This has indeed been demonstrated for IL-2 in human T-lymphocytes, where a combination of both drugs has a much greater suppressive effect than either drug alone [176]. This suggests that a dose of cyclosporin A that is too low to give nephrotoxic side effects may be combined with an inhaled steroid, so that this synergistic interaction is confined to the airways.

Another interaction that may be exploited therapeutically is that between retinoic acid and steroids. Retinoic acid (vitamin A) binds to retinoic acid receptors which, like GR, bind to CBP. There appears to be a synergistic interaction between steroids and retinoic acid in repression of transcription factors such as NF-κB and AP-1, presumably because of competition for binding sites on CBP. A synergistic interaction between retinoic acid and steroids has been demonstrated in suppression of GM-CSF release from cultured epithelial cells, suggesting that retinoic acid may potentiate the anti-inflammatory effects of steroids [177]. Novel retinoic acid derivatives activate a subtype of retinoic acid receptor (RXR) which interacts with these transcription factors, and thus it may be possible to develop more selective retinoids for this purpose [178].

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Exhibit G

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Review

The NOD Mouse as a Model of SLE

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In addition to developing a high incidence of type 1 diabetes caused by a specific autoimmune response against pancreatic β cells in the islets of Langerhans, NOD mice also demonstrate spontaneous autoimmunity to other targets including the thymus, adrenal gland, salivary glands, thyroid, testis, nuclear components and red blood cells. Moreover, treatment of pre-diabetic NOD mice with an intravenous dose of heat killed *Mycobacterium bovis (M. bovis*; bacillus Calmette-Guèrin (BCG)) protects them from developing type 1 diabetes, but instead precipitates an autoimmune rheumatic disease similar to systemic lupus erythematosus (SLE), characterised by accelerated and increased incidence of haemolytic anaemia (HA), anti-nuclear autoantibody (ANA) production, exacerbation of sialadenitis, and the appearance of immune complex-mediated glomerulonephritis (GN). The reciprocal switching between the two phenotypes by a single environmental trigger (mycobacterial exposure) raised the possibility that genetic susceptibility for type 1 diabetes and SLE may be conferred by a single collection of genes in the NOD mouse. This review will focus on the genetic components predisposing NOD mice to SLE induced by BCG treatment and compare them to previously determined diabetes susceptibility genes in this strain and SLE susceptibility genes in the BXSB, MRL and the New Zealand mouse strains.

Keywords: NOD mice, SLE

INTRODUCTION

The coexistence of systemic lupus erythematosus (SLE) and type 1 diabetes in human individuals is rare, with its frequency not deviating significantly above that expected if the inheritance of the two diseases were completely independent. However, there is evidence to suggest that the two diseases may share a common genetic aetiology. For instance, sixteen percent of patients with type 1 diabetes have been found to develop anti-nuclear autoantibodies (ANA) compared to only one percent of normal controls[1], and major histocompatibility complex (MHC) haplotypes which confer susceptibility to SLE, such as B8,SC01,DR3,DQ2,Dw24 and B18,F1C30,DR3, DQ2, Dw25, have also been shown to predispose to

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type 1 diabetes[2]. Furthermore, six of the human type 1 diabetes susceptibility loci (*IDDM2, IDDM3, IDDM7, IDDM8, IDDM11, IDDM12*) have been mapped to similar genomic positions as those recently reported in linkage studies of SLE[3]. Concannon *et al.*[4] have also reported a novel type 1 diabetes susceptibility locus on chromosome 1q42 which maps to a similar position as a prominent SLE susceptibility locus in this region[5–8]. The clustering of susceptibility loci between these two diseases has also been observed in mouse models, with six colocalisations (including the MHC) occurring between the genes conferring susceptibility to type 1 diabetes in non-obese diabetic (NOD) mice and those predisposing to SLE in the BXSB, MRL and the New Zealand strains of mice on chromosomes 1, 4, 6, 7 and 17 (Figure 1). Moreover, the lupus prone NZB, (NZB × NZW)F1 and MRL mice develop spontaneous autoimmune reactions against pancreatic islets[9] and NOD mice develop lupus-like disease in senescence[10,11], which can be increased in severity and accelerated by treatment with heat killed *M. bovis*[12].

In addition to the evidence presented for type 1 diabetes and SLE, genetic studies in both humans and mice have begun to unveil a substantial genetic colocalisation/overlap between the susceptibility genes of various distinct autoimmune diseases[3,13,14]. Recently, Becker *et al.*[15] performed a comprehensive controlled meta-analysis comparing the results of linkage analyses in twenty three autoimmune diseases and immune-mediated diseases in humans and animal models. The study revealed that apart from the MHC, 65% of positive linkages mapped non-randomly into eighteen distinct clusters throughout the human genome. These clusters identified by Becker and colleagues may represent areas of the genome in which genes controlling immune function have aggregated by gene duplication and/or selective advantage, in a similar manner to those of the MHC. On the other hand, it may also be possible that particular alleles of a gene or genes within these loci confer susceptibility to several autoimmune diseases and thus contribute to autoimmunity *per se*.

Perhaps the strongest evidence for the existence of common autoimmune disease susceptibility genes was provided when NOD mice congenic for the diabetes resistant *Idd3* allele (0.15cM interval) of the C57BL/10 mouse, were shown to have a decreased severity and duration of experimental autoimmune encephalomyelitis (EAE) compared to wild type NOD mice[16]. Since an EAE susceptibility gene (*Eae3*) is located in a similar area of the mouse genome as *Idd3*[17,18], it is possible that the same gene may be conferring susceptibility to both diseases. Indeed, both NOD and SJL (a commonly used EAE-prone strain) share the same allele of the most attractive candidate gene in this interval, *Il2*. In addition, an autoimmune ovarian dysgenesis (AOD) susceptibility locus (*Aod2*) in mice also colocalises with *Idd3*, and the disease susceptible strain, A/J, shares the same *Il2* allele as NOD[19]. Conversely, all of the disease resistant mouse strains used in the above mapping studies share a different allele of *Il2*[16].

The interaction of these common autoimmunity genes with other genes, environment and/or stochastic factors may determine the eventual specificity/specificities of the autoimmune response, resulting in the development of one or more autoimmune diseases.

SLE INDUCED BY BCG TREATMENT IN NOD MICE

The NOD mouse strain is a widely used model of type 1 diabetes. However, in addition to a specific autoimmune response against pancreatic β cells in the islets of Langerhans, NOD mice also demonstrate spontaneous autoimmunity to other targets including the thymus[20], salivary glands, thyroid, adrenal, testis[21], nuclear components[10] and red blood cells[11]. Of these, only the targeting of red blood cells results in disease, causing haemolytic anaemia (HA) in these mice[11]. NOD mice only develop HA spontaneously in their old age (>250 days), with approximately 80% of mice surviving more than 400 days (i.e. non-diabetics) developing this disease[11]. Overall, these results imply a generalised defect in the process of immunological tolerance in the NOD mouse. This defect in the NOD can ultimately result in the expression of various autoimmune phenotypes depending on the interaction of the genetics with environmental or stochastic factors.

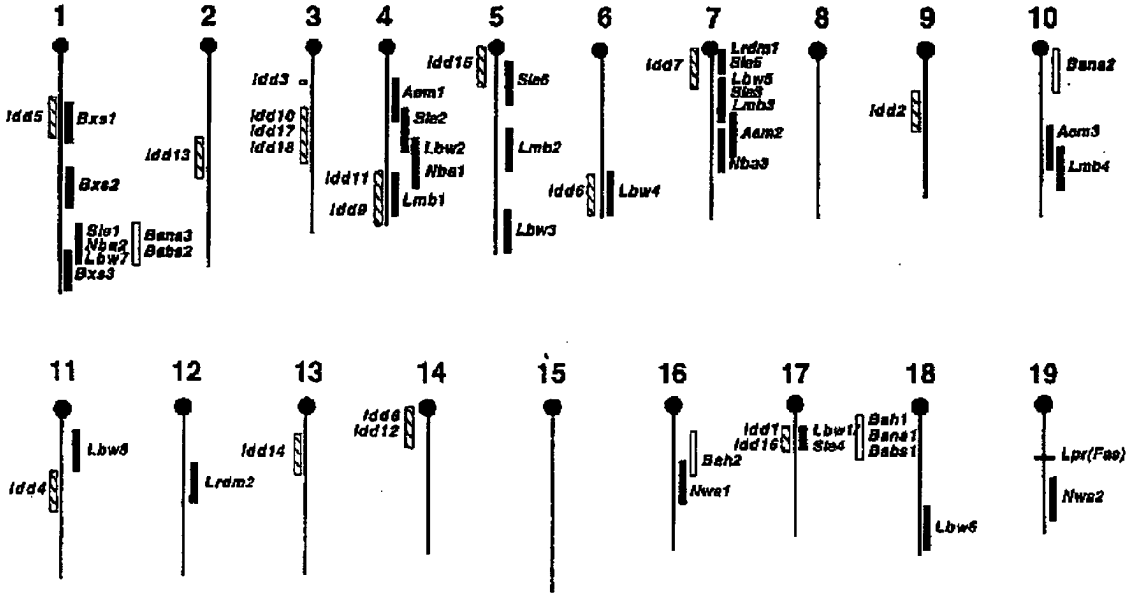

FIGURE 1 Comparing the positions of BCG induced SLE susceptibility loci to susceptibility loci from spontaneous SLE models and type 1 diabetes. Type 1 diabetes loci are designated Idd and are represented by bars containing diagonal lines[16,24–26,31–34,40–46,84,93]. All SLE loci are represented by black bars. Loci from NZB/NZW crosses are designated Nba, Sle, and Lbw[53,57,59,73,79,85,86]. HA loci from NZB crosses are designated Aem[87]. Loci from BXSB crosses are designated Bxs[60,88]. Loci from MRL-lpr crosses are designated Lpr, Lmb, and Ldrm[80,89–91]. Loci derived in the study of BCG-induced lupus are represented by white bars and are designated Bah, Bana, and Babs[47]

In mice, two of the genes which control the strength of the immune response to intracellular pathogens have been mapped to chromosome 1 (*solute carrier family 11 member* 1 (*Slc11a1*) gene, formerly *Bcg/Lsh/Ity*) and 17 (*H2*), respectively[22,23]. Interestingly, these loci map to similar positions as the diabetes susceptibility genes *Idd5* and *Idd1/16* in the NOD mouse[24–26]. Injection of heat killed *Mycobacterium bovis* (*M. bovis*; bacillus Calmette-Guèrin (BCG)) into NOD mice protects them from diabetes, establishing a link between the disease and mycobacteria[27]. In order to examine the role of non-NOD alleles at the *H2* on the responses of NOD mice to BCG, Baxter *et al.*[12] injected NOD and NOD.E^+transgenic mice with the vaccine. As previously determined, the BCG treatment of the NOD controls protected them from the development of diabetes. However to their surprise, they discovered that two to three months after the BCG treatment, the control mice developed an autoimmune rheumatic disease similar to SLE[12]. The manifestations precipitated by the BCG treatment in NOD mice included: (1) the development of HA in half of the mice much earlier than would have occurred spontaneously (150 days vs. >250 days); (2) increase in the severity of sialadenitis; (3) hypergammaglobulinaemia; (4) greatly elevated and accelerated production of anti-nuclear autoantibodies (ANA) in the majority of mice, binding primarily to nuclear proteins such as Sm antigen, but also to double stranded (ds) DNA; and (5) extensive immune complex deposits in glomeruli and C3c activation, consistent with glomerulonephritis ((GN); [12,28,29]). The diverse nature of antigens targeted in this disease suggests that the mycobacterial antigens in the BCG vaccine were unlikely to be cross-reactive with the autoantigens mediating pathology. On the contrary, we have proposed an alternative adjuvant-like mechanism[29].

The SLE induced by BCG in the NOD mouse has some unique features and some characteristics in common with other mouse models of SLE including the MRL/*lpr*, (NZB × NZW)F1 and BXSB

strains[12,28,30]. For example, elevated serum immunoglobulin levels (hypergammaglobulinaemia) and anti-dsDNA are common to all of the murine models of SLE. The presence of anti-erythrocyte autoantibodies and haemolytic anaemia are shared with the NZB and (NZB × NZW)F1 strains, but absent in the MRL and BXSB strains. Despite being an important diagnostic criterion of human SLE, high titres of autoantibodies directed against the Sm antigen are only found in MRL/lpr and NOD mice. Salivary gland periductal mononuclear infiltrates (sialadenitis), which occur with an increased severity in BCG treated NOD mice, have also been reported in MRL/*lpr* and, to a lesser extent, in (NZB × NZW)F1 mice. Unlike that in NZB/W mice, the pattern of renal disease in NOD mice treated with BCG is similar to mild focal lupus nephritis (WHO classification IV) in humans, and is characterised by widespread C3c deposition and segmental proliferation of a minority of glomeruli. BCG treatment of normally non-autoimmune mouse strains such as BALB/c, C57BL/6, CBA and DBA, did not cause the development of the SLE manifestations seen in the NOD mouse[29]. Therefore, the genetic background of the NOD mouse is likely to play a critical role in this systemic autoimmune disease. As a model of SLE, NOD mice offer two main advantages for studying the genetics of autoimmune disease. Firstly, as the BCG-induced SLE model is one of the few models of autoimmune disease where the environmental trigger has been identified, it provides an excellent opportunity for studying the genetic/environmental interactions required to initiate autoimmunity. Thus it was of specific interest to determine if the genes which mediate protection/susceptibility to intracellular pathogens (such as *Slc11a1* and *H2* genes), also play a role in controlling susceptibility to autoimmune disease induced by infectious agents such as mycobacterium. Secondly, the development of SLE and type 1 diabetes within the same mouse strain, with reciprocal switching between the two phenotypes induced by an environmental trigger, raises the possibility that both autoimmune diseases could be controlled by a single set of genes. That is, NOD mice inherit a single disease (autoimmunity), which can be expressed as at least two phenotypes (SLE or diabetes), depending on environmental (e.g. mycobacterial) or stochastic factors. The genetic loci conferring susceptibility to type 1 diabetes in the NOD mouse have been extensively characterised by various groups[16,19,24–26,31–46,93]. By mapping the positions of the genes conferring susceptibility to SLE on the NOD genetic background, a direct comparison of the genetic determinants in both autoimmune diseases can be performed. In addition, susceptibility loci found in this study may also be compared with those of other autoimmune diseases in mice and humans in light of this common autoimmune gene hypothesis.

THE GENETICS OF SLE IN THE NOD MOUSE

A linkage analysis study was performed by Jordan *et al.*[47] in order to map the genes conferring susceptibility to SLE induced by BCG in NOD mice, with a special interest in those genes which may colocalise with type 1 diabetes loci detected in the same strain. As a cross between NOD and C57BL/6 mice was initially precluded due to the high incidence of GN induced by BCG in C57BL/6 and F1 mice, a backcross between NOD and BALB/c mice was used for the genetic study instead. Four SLE-related traits were analysed independently in the backcross mice, including HA, production of ANA, production of autoantibodies (i.e. Coombs' antibodies and/or ANA) and immune complex-mediated GN (summary of the results is illustrated on Figure 1). The strongest linkage to HA, ANA and autoantibody production in the study (genes designated *Bah1*, *Bana1* and *Babs1*, respectively) was exhibited by the NOD *H2* region ($H2^{g7}$). This finding was consistent with the critical role of the *H2* in the development of SLE in other strains of mice including the (NZB × NZW)F1 and the BXSB. Furthermore, the NOD $H2^{g7}$ haplotype is also the major determinant of diabetes susceptibility in the NOD mouse (designated *Idd1/16*; [24,26,31,32]). Thus, in an analogous fashion to the B8,SC01,DR3,DQ2,Dw24 and B18,F1C30,DR3, DQ2, Dw25 haplotypes in humans, the $H2^{g7}$ haplotype in NOD mice contributes to the susceptibility to both diabetes and SLE. This finding provides further

support for the common gene hypothesis, at least in regards to the MHC.

The most attractive candidate genes at the *Bana1/Bah1/Babs1* region are the class II MHC genes, *E* and *A*. These genes have demonstrated a strong association with SLE (especially with the production of autoantibodies) within the MHC region in humans[48,49]. In mice, the role of the I-A molecule in SLE was best demonstrated in an experiment involving NZB. $H2^{bm12}$ and NZB. $H2^{b}$ congenic mice[50]. Despite only differing at three amino acids at the peptide binding groove of the I-A molecule, these mice exhibit large differences in their susceptibility to SLE. BXSB mice, on the other hand, which do not express I-E molecule (share the E^{b} allele with NOD mice), were protected from the development of SLE by the introduction of a transgene encoding the I-Eα molecule onto their genetic background[51]. This protection may be mediated by the same mechanism which protects NOD mice expressing the I-Eα transgene from diabetes (NOD. E^{+}; [52]). To determine if the absence of the I-E molecule also confers susceptibility to SLE in NOD mice, the next step will involve the production and testing of I-E expressing congenic or transgenic NOD mice for the development of lupus traits after BCG treatment. In addition to being associated with the development of several autoimmune diseases, the *H2* region regulates the vigour of immune responses towards intracellular pathogens[23]. It is thus also possible that genes within the MHC region may regulate the expression of autoimmune disease in the NOD mouse through an interaction with *M. bovis*.

Suprisingly, no further colocalisation between the genes conferring susceptibility to SLE in the NOD/BALB backcross and the previously determined diabetes susceptibility genes were found, except perhaps for a few putative linkages to GN which mapped close to two diabetes susceptibility loci (discussed below). This result would imply that the non-MHC susceptibility genes affecting type 1 diabetes and SLE in NOD mice are distinct, and thus, are likely to affect distinct biological pathways leading to autoimmunity. In this case, the environmental agent, *M. bovis*, would regulate the expression of these two sets of genes in a way that would favour the development systemic autoimmunity over organ specific autoimmunity. Nevertheless, it is unlikely that all of the non-MHC genes predisposing to SLE in NOD mice were detected in our cross. It has become apparent from several studies that contributions from certain disease susceptibility loci (including the MHC) can differ markedly depending on the non-autoimmune strain used in linkage studies[33,53]. This phenomenon may be caused by epistatic interactions of susceptibility genes with the non-autoimmune genetic background and/or the sharing of disease mediating alleles between strains. The majority of NOD diabetes susceptibility loci were mapped using a NOD/C57BL/10 or NOD/C57BL/6 combination[54]. However, although it would have been ideal to use the NOD and C57BL/6 strains for detecting common SLE and diabetes genes, this strain combination was not used due to the high level of immune-complex mediated GN occurring in their F1 progeny.

Outside the MHC, *Idd3* on chromosome 3 has a large influence on the development of type 1 diabetes in NOD mice. In addition, *Idd3* has shown evidence of being a common autoimmunity gene, as it also confers susceptibility to EAE in NOD mice[16]. A polymorphism within exon 1 of the NOD *Il2* gene seems to consistently segregate with *Idd3* in several mouse crosses, making it the major candidate within this region[19]. Chestnut *et al.*[55] have sequenced *Il2* in several mouse strains and found that C57BL/10, C57BL/6 and BALB/c all share an allele of this gene which is associated with resistance to both diabetes and EAE. Therefore, if *Il2* is indeed *Idd3*, the fact that no linkage was detected on chromosome 3 for any of the SLE traits tested in this study signifies that this promising common autoimmunity gene is unlikely to affect systemic autoimmunity in NOD mice.

A particularly interesting finding in the Jordan *et al.*[47] study was the linkage detected for ANA and autoantibodies on distal chromosome 1 (designated *Bana2* and *Babs3*, respectively). These loci mapped to a similar region as a cluster of SLE susceptibility loci derived from the BXSB, NZB/NZW and NZM2410 strains (Figure 1) which include: *Lbw7* and

Nba2, both NZB derived genes affecting the production of ANA and GN[53,56,57]; *Sle1*, an NZW-derived susceptibility gene which also confers susceptibility to GN and ANA[58,59]; and *Bxs3*, a BXSB susceptibility gene associated with ANA production[60]. Furthermore, the syntenic region in humans (1q23–24) has also exhibited linkage to SLE in several diverse populations, including Caucasian, African-American and Mexican-American[5,6]. The detection of linkage to SLE within this segment in various mouse strains and multiple human ethnic groups implies the presence of a gene, or a cluster of genes, which have a potent effect on the development of systemic autoimmunity.

Two very promising candidate genes within the *Bana3/Babs2* interval on chromosome 1 include *Fcgr2b* (encoding Fc gamma receptor IIB (FcγRIIB; CD32)) and *Fasl* (encoding Fas ligand). While most other FcγR molecules promote inflammatory responses when coligated by IgG immune complexes, FcγRIIB downregulates the activation of cells such as B lymphocytes, monocytes and macrophages, due to an ITIM motif in its cytoplasmic domain[61]. The targeted deletion of *Fcgr2b* in mice leads to an enhanced production of immunoglobulin by B cells in response to antigen[62]. Furthermore, a destructive inflammatory response, mainly mediated by macrophages, can also be induced in *Fcgr2b*$^{-/-}$ mice when injected with concentrations of immune complexes that have no effect on wild type mice[63]. Yuasa *et al.*[64] have also demonstrated that the targeted deletion of *Fcgr2b* in the normally non-autoimmune 129/C57BL/6 (*H2*^{b}) hybrid strain predisposed these mice to collagen induced arthritis, associated with a large increase in IgG autoantibody levels specific for collagen. In the studies described above, the primary cause of the enhanced humoral and inflammatory responses in *Fcgr2b*$^{-/-}$ mice was shown to be due the decreased threshold of activation displayed by macrophages and B cells when they bound immune complexes. Interestingly, a recent report has demonstrated that the promoter region of the *Fcgr2b* gene of NOD mice contains a deletion which results in the reduced cell surface expression of FcγRIIB molecules on macrophages and activated B cells[65]. This promoter polymorphism is also shared by a number of lupus prone mouse strains including NZB, BXSB and MRL[65,66]. On the other hand, the polymorphism was absent in autoimmune resistant mouse strains such as BALB/c, C57BL/6, C57BL/10 and DBA/2.

Fas ligand is very tightly linked to the marker of highest linkage (*D1Mit399*) in both the ANA and autoantibody linkage datasets. This molecule, expressed mainly on activated T cells and NK cells, is a member of the tumour necrosis factor receptor family and induces apoptotic death in Fas bearing target cells[67]. Mice with a natural deficiency in Fas ligand due to a mutation in the *Fasl* gene (termed the *gld* mutation), develop systemic autoimmunity and a progressive accumulation of abnormally activated lymphocytes[68,69]. The severity of lupus-like disease is dependant on the genetic background of the mice carrying the *gld* mutation, with severely affected strains developing acute renal disease, vasculitis and arthritis[69]. Several studies utilising Fas- and FasL- deficient mice (i.e. carrying the *lpr* and *gld* mutations respectively) have revealed that these molecules are likely to be important in the maintenance of peripheral tolerance in lymphocytes[70]. Thus defects in this molecule could predispose NOD mice to the development of autoimmunity. Restriction fragment length polymorphism (RFLP) analysis of *Fasl* has shown that there are two alleles of this gene in the mouse, *mFasL.1* and *mFasL.2*, which are distinguished by two amino acid substitutions which are predicted to occur in the Fas binding domain of the molecule[71]. Functional analysis revealed that the protein encoded by *mFasL.1* has nine fold higher cytotoxic activity than that of *mFasL.2*, regardless of the allotype of Fas molecule displayed by target cells. Interestingly, the *mFasL.2* is present in several autoimmune strains including NOD, MRL, NZB, SJL, C3H and NZW, while BALB/c, DBA/1 and DBA/2 express mFasL.1[71].

Despite being used as the resistant strain in the backcross with NOD mice, the linkage analysis of SLE induced by BCG also revealed two BALB/c derived genes designated *Bah2* (chromosome 16) and *Bana2* (chromosome 10), which confer susceptibility specifically to HA and the production of ANA, respectively. Other autoimmune disease susceptibility

loci have been detected on the BALB/c genetic background including *Gasa1* and *Gasa2* on chromosome 4[72] predisposing to experimental autoimmune gastritis (EAG) and the *Baa* locus on chromosome 9, which is an SLE modifier[73]. *Bana2* and *Bah2* displayed decreased linkage in the combined analysis of autoantibodies, suggesting that their effects were diluted by the addition of mice containing differing autoimmune specificities. It is therefore likely that these genes are involved in directing the specificity of the autoimmune response against certain autoantigens (i.e. red blood cells or nuclear antigens).

It was noticed that *Bah2* mapped to a similar position as *Aod1*, a locus found to confer susceptibility to neonatal thymectomy induced AOD in the A/J strain. As female BALB/c mice develop AOD after d3Tx at a low incidence (20–30%; [74]), it is possible that *Bah1* and *Aod1* represent a common autoimmunity gene between HA and AOD[75]. Interestingly, the genes of the immunoglobulin lambda light chain are contained within the *Bah2/Aod1* region of chromosome 16[76]. This cluster of genes are thus proposed as candidates for *Bah2* since they could fulfil the role of being involved in two diseases while still being able to direct the specificity of an autoimmune response. Indeed, a limited number of association studies in humans have found that polymorphisms within the variable genes of the immunoglobulin heavy chain influence the development of SLE[77,78].

The development of GN after BCG treatment had an apparent genetic aetiology due to the high incidence of the trait in the NOD mice (90%) and the reduced incidence in the BALB/c and C57BL/6 strains (30% and 44%, respectively; [47]). However, significant linkage to GN was not found in the SLE linkage study performed by Jordan and colleagues. It is likely that the existence of some background GN in the BALB/c strain after BCG treatment may have reduced the power of this linkage study. Nevertheless, various loci displaying a suggestive level of linkage were detected on chromosomes 1, 4, 12, 16 and 17, implicating a number of putative susceptibility genes. The locus on chromosome 4 generated some interest as it was mapped to a similar region as several GN susceptibility loci derived from NZB, NZW and MRL mouse strains including *Nba1*, *Lbw2* (both NZB derived; [57,79]), *Sle2* (contains NZB and NZW contributions; [59]) and *Lmb1* (C57BL/6-*lpr* derived; [80]). This interval also maps close to the NOD diabetes susceptibility gene *Idd11*[44]. Similarly, the putative susceptibility locus on proximal chromosome 1 is mapped near a BXSB GN susceptibility locus (*Bxs1*) and a NOD diabetes susceptibility locus (*Idd5*; [25,60]). These colocalisations could indicate shared susceptibility genes between these traits. One of the candidate genes for the proximal chromosome 1 region is the *Slc11a1* gene, which codes for a macrophage-specific membrane transport protein that determines susceptibility to intracellular pathogen infection[22]. This candidate gene would be consistent with GN being the trait most affected by BCG treatment, since few NOD mice develop this manifestation spontaneously in senescence compared to the development of ANA or HA[28]. Polymorphisms in the *Slc11a1* gene have been detected between the NOD and BALB/c strains, which could ultimately affect susceptibility to GN through altered interactions with BCG[81]. Despite mapping to interesting positions in the genome, both of the putative GN susceptibility loci on chromosome 1 and 4 require confirmation in an independent linkage study. Alternatively, the effects of these loci on GN susceptibility could be checked by immunising NOD.*Idd5*^{r} or *Idd11*^{r} congenic mice with heat killed *M. bovis*.

In addition to the loci detected through linkage analysis, the examination of different directions of backcrosses between the NOD and BALB/c mice revealed the likely existence of an SLE susceptibility gene/s on the X chromosome which affected the development of HA and GN, but not ANA (Table I). The possibility that HA and GN share or have closely linked susceptibility genes on the X chromosome may account for the unusually high number of backcross mice which were found to develop both of these phenotypes[47]. Reciprocal backcrossing of NOD with various outcross partners, including C57BL/10 and NON, has not demonstrated the presence of a type 1 diabetes susceptibility loci on the X chromosome[33,34]. Thus this gene on the X chromosome may be SLE specific. However, whether this gene segregates with diabetes in the NOD/BALB strain combination is yet to be tested.

TABLE I Incidences of SLE associated traits after BCG treatment in different directions of backcrosses[a]

Direction of Backcross (Female × Male)	*X specific genes*	*HA incidence*[b]	*ANA incidence*[c]	*GN incidence*[d]
NODx(NODxBALB)	NOD homozygous	14/207 (7%)	23/217 (11%)	25/218 (13%)
NODx(BALBxNOD)	NOD/BALB heterozygous	1/163 (0.6%)	14/164 (9%)	13/293 (4%)
(NODxBALB)xNOD & (BALBxNOD)xNOD	Segregating: NOD or NOD/BALB	21/496 (4%)	97/420 (23%)	34/488 (7%)

[a]Data source from [47].

[b]NODx(NODxBALB) vs. NODx(BALBxNOD) incidence; $p < 0.01$, fourfold table χ^2 test.

[c]NODx(NODxBALB) vs. NODx(BALBxNOD) incidence; NS, fourfold table χ^2 test.

[d]NODx(NODxBALB) vs. NODx(BALBxNOD) incidence; $p < 0.01$, fourfold table χ^2 test.

TABLE II Observed GRR for pairs of susceptibility loci versus predicted values for additive and multiplicative interaction models[a]

Trait	*Locus 1 (GRR)*	*Locus 2 (GRR)*	*Observed*					*Additive*		*Multiplicative*	
			A_{22}	A_{21}	A_{12}	A_{11}	*GRR*[b]	*GRR*	χ^2	*GRR*	χ^2
HA	Bah1 (0.59)	Bah2 (2.83)	4	25	2	3	2.13	0.88	12.56	1.66	0.84
ANA	*Bana1* (0.70)	*Bana3* (0.68)	29	8	9	6	0.45	0.60	6.36	0.48	0.88
ANA	*Bana1* (0.70)	*Bana2* (1.73)	8	29	7	8	1.63	1.00	7.89	1.22	1.56
ANA	*Bana3* (0.68)	*Bana2* (1.73)	13	25	2	12	1.00	0.96	0.08	1.19	1.09
AAB	*Babs1* (0.65)	*Babs2* (0.73)	49	21	15	8	0.47	0.58	4.78	0.48	0.09

[a]Data source from [47].

[b]The genotype risk ratio (GRR) is the ratio of disease risk in the backcross progeny versus the disease risk in the susceptible parental strain, due to segregation (homozygous versus heterozygous) at a susceptibility locus or set of susceptibility loci. The GRR was calculated using the formula derived by Risch *et al.*[92]: $GRR = A/2^n$. A_n, where A is the total number of affected backcross mice in the dataset, n is the number of loci being tested and A_n is the number of affected backcross mice carrying the genotype from the susceptible mouse strain at the locus/loci being tested. To characterise the relationship amongst pairs of susceptibility loci, the observed GRR calculated for two loci were compared to expected GRR values of an additive (lack of epistasis) and multiplicative (presence of epistasis) model of interaction as defined by Risch and colleagues.

One of the unexpected results in this study was the lack of association between the development of ANA and GN in the NOD/BALB backcross panel. These data contradicted the proposed causative role of ANA (especially anti-dsDNA autoantibodies) in the development of glomerulonephritis[32]. Nevertheless, the independent segregation of these traits has also been reported in the progeny of a backcross between NZB and NZW mice and F2 intercrosses involving the NZM2410 and C57BL/6 strains, suggesting distinct genetic contributions are probably responsible for the development of these traits[59,83]. Indeed, the current study did not find any evidence of common susceptibility loci between ANA and GN. Although both HA and ANA showed strong linkage to the *H2* region, the coinheritance of these traits was not higher than that expected by chance. This may reflect a requirement for epistatic interactions between the MHC genes and distinct HA and ANA-specific non-MHC genes. Indeed this seems to be the case as the combined genotype risk ratios (GRR) of MHC and non-MHC susceptibility genes were found to conform better to the multiplicative model of interactions rather than the additive model (Table II).

CONCLUSION

In NOD mice, genes within two genomic regions on chromosome 1 (*Bana3/Babs2*) and 17 (*Bah1/Bana1/Babs1*) appear to be of great importance to the devel-

opment of SLE and autoimmune disease. The *Bah1/Bana1/Babs1* genes, which are probably within *H2*, colocalise with the *Idd1/Idd16* genes predisposing to diabetes on the same genetic background and thus may represent common autoimmunity gene/s affecting both diseases within the MHC. The *Bana3/Babs2* genes are located in a similar position as several SLE susceptibility loci from other mouse models and are syntenic to an SLE susceptibility locus present in various human populations. The colocalisation/synteny of these SLE genes may indicate the presence of a gene (or cluster of genes) with a potent effect on systemic autoimmunity in this region. The elucidation of these NOD susceptibility genes, through the development of congenic mice, candidate testing and/or positional cloning, will no doubt be important for developing a greater understanding of the mechanisms responsible for initiating SLE and autoimmune diseases in general.

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The Murine Autoimmune Diabetes Model: NOD and Related Strains

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I. Introduction

Insulin-dependent diabetes mellitus (IDDM, type I diabetes) is a metabolic disease caused by insulin deficiency. In this disease, mononuclear infiltration is observed in pancreatic islets, and insulin-producing β cells are selectively destroyed, resulting in insulin deficiency. Other pancreatic endocrine cells, such as glucagon or somatostatin-producing cells, are not affected. Mononuclear cell infiltration is observed in pancreatic islets of IDDM patients. Furthermore, autoantibodies to pancreatic islet cells are usually detected even before the onset of disease. Therefore, IDDM is considered as one of typical organ-specific autoimmune diseases. Apparent symptoms show up suddenly when most of the pancreatic β cells are irreversibly destroyed after a long, chronic, autoimmune inflammatory process. This sometimes makes it difficult to look at an initial process of human IDDM. Genetic susceptibility appears to affect the development of IDDM. Population studies as well as studies of families of patients indicate that the development of IDDM is associated with genes linked to the major histocompatibility complex (MHC), particularly the class II region. Recent advances in HLA typing have contributed very much to indentifying the class II haplotypes that determine the disease susceptibility. The haplotypes identified in IDDM patients are also found in normal individuals. Furthermore, the concordance of IDDM in monozygotic twins is approximately 50%. These data suggest that environmental factors and genes other than those of the MHC also influence the development of IDDM. In fact, environmental factors affecting IDDM induction may include diabetogenic viruses, diet, and β cell-tropic toxins.

To elucidate the etiology of IDDM, several animal models have been developed, including experimentally induced and spontaneously developing models. One is low-dose streptozocin-induced diabetes. A high dose of streptozocin destroys completely pancreatic β

cells but not α and δ cells, resulting in acute diabetes (Rakieten *et al.*, 1963). Multiple injections of subdiabetogenic doses of streptozocin can also induce diabetes a few weeks after treatment (Like and Rossini, 1976). In the latter case, mononuclear infiltration in and around pancreatic islets is observed. This indicates that diabetes in low-dose streptozocin-treated animals is caused by an inflammatory process that follows partial destruction of pancreatic islets rather than the drug-induced direct destruction. In human IDDM, viral infection of an unknown nature is also one of the candidates for environmental diabetogenic factors. Certain viruses are known to induce diabetes in rodents. However, it is still controversial whether virus-induced IDDM in animal models is a consequence of a direct cytotoxic effect on, or an immunologically mediated destruction of, pancreatic β cells. Although these experimentally induced models have been based on the assumption that environmental etiologic factors play a major role in an initiation of IDDM, it is unknown whether these models really represent spontaneously developing IDDM in humans. Nakhooda *et al.* (1977) have reported spontaneously developing IDDM in the BB rat, a Wistar-derived rat. IDDM occurs in approximately 60% of BB rats between 60 and 120 days of age (Nakhooda *et al.*, 1978). Mononuclear cell infiltration in and around pancreatic islets always precedes complete destruction of β cells and overt diabetes (Logothetopoulos *et al.*, 1984). Immunological studies indicate that insulitis observed in BB rats is mediated by an autoimmune process. The development of IDDM in BB rats is closely linked to the class II region of the MHC (Colle *et al.*, 1981, 1986). In 1974, a mouse exhibiting polyuria, severe glycosuria, and rapid weight loss was discovered in Japan and the nonobese diabetic (NOD) mouse strain was established a few years later (Makino and Tochino, 1978; Makino *et al.*, 1980). IDDM in NOD mice is also immunologically mediated. IDDM in BB rats and NOD mice is very similar to the disease in humans. The availability of a large number of data and materials in mouse genetics and immunology has accelarated the use of NOD mice by diabetologists and immunologists who are interested in the pathogenesis of IDDM and the mechanism of autoimmunity. In fact, several important findings obtained from studies on NOD mice have provided new insights into the immunological and genetic control of IDDM.

In this review, we summarize the experimental findings and observations based on the NOD mouse model, and we discuss on the pathogenesis and the etiology of autoimmune diabetes.

II. Development of the NOD Strain

The discovery of a female mouse exhibiting polyuria, severe glycosuria, and rapid weight loss in 1974 triggered the development of the NOD strain. At that time, many animal models for diabetes mellitus had already been reported (Herberg and Coleman, 1977; Herberg, 1979). Most of them were, however, models for type II diabetes mellitus. A suitable animal model for type I diabetes mellitus had been desired for a long time. Diabetic symptoms without obesity exhibited by the female mouse were an attractive inducement for researchers to attempt the development of such a new diabetic mouse strain. Selective breeding using the offspring of the female mouse was performed, and the NOD strain was established in 1980 in Shionogi Aburahi Laboratories as an animal model for type I diabetes mellitus (Makino *et al.*, 1980).

The pedigree of the diabetic female mouse is shown in Fig. 1. In 1966, a female mouse exhibiting cataracts and small eyes was found among outbred ICR mice purchased from Clea Japan, Inc., and the inbred cataract Shionogi (CTS) strain with the same characteristics and its control strain, the noncataract (NCT) strain, were established (Ohotori *et al.*, 1968; Makino *et al.*, 1989). Since cataracts are often observed in diabetic patients, an attempt at selective breeding for euglycemic and hyperglycemic lines was made at the sixth generation

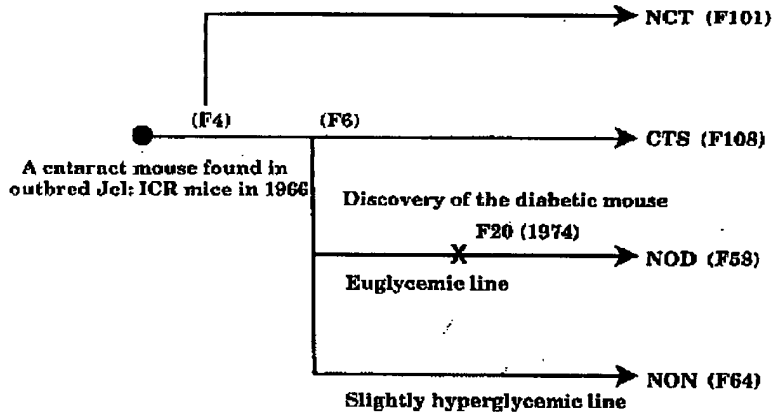

FIG. 1. Pedigree of the NOD mouse and related strains. All four strains have been established from an outbred colony of Jcl:ICR.

during the process of establishing the CTS strain. Two sublines, one euglycemic and one slightly hyperglycemic, were separated after selective breeding for 13 generations. The diabetic female mouse spontaneously arose at the twentieth generation of the former line.

For the selective breeding of mice with glycosuria, the initial eight mating pairs were composed using the offspring of the two sublines. Eighteen litters consisting of 66 females and 72 males were obtained from the initial mating pairs. Of these F_1 mice, 12 females and 3 males developed the same diabetic symptoms. Only the progenies having one or both parents spontaneously developing glycosuria were selected for the next generation, and the process was repeated. Thus, three diabetic lines were produced. One of these lines, in which the mice showed good reproductivity, was selected as the main diabetic line and named the NOD strain (Makino *et al.*, 1980). However, a control stain for the NOD strain could not be separated from the three diabetic lines, so another line was developed from the aforementioned slightly hyperglycemic line and named the nonobese nondiabetic (NON) strain.

The animals were maintained under conventional conditions until the fourth or fifth generations, and were thereafter transferred by cesarean operation under barrier conditions free from mouse hepatitis virus (MHV), Sendai virus, Hantaan virus, *Mycoplasma, Corynebacterium, Bordetella, Bacillus piliformis, Pasteurella, Pseudomonas aeruginosa,* and *Staphylococcus aureus.* However, the onset and the symptoms of diabetes were the same as those of mice maintained under conventional conditions.

The genetic constitutions of the NOD, CTS, NCT, and NON strains were studied at the 25 loci on genes encoding coat color, biochemical markers, and immunological markers on the 13 chromosomes (Makino *et al.*, 1989). None of the strains showed heterozygous genotypes in any of the markers. Among the strains, however, differences were observed at some markers, i.e., the *a, Idh-1, Amy-2, Car-2, Pgm-1, Gpi-1, Mod-1, Ly-2, Ly-3, Thy-1,* and MHC genes. Therefore, from a genetic aspect, the NOD, CTS, NCT, and NON strains are each pure inbred mice, but they are mutually independent strains. Their present inbreeding generations are F_{58}, F_{107}, F_{101}, and F_{64}, respectively.

The NOD/Shi colony has shown diabetes with constant incidences of 70–80% for females and of 20% for males from the early generations to the present generation. However, differences in the diabetes incidences among NOD colonies have recently been indicated: i.e., the NOD/WEHI colony shows a low incidence, less than 10% of females (Baxter *et al.*, 1989), whereas the NOD/Alt colony shows a high incidence, over 95% of females (Sadelain *et al.*, 1990). Although environmental factors such as diet (Elliott *et al.*, 1988; Coleman *et al.*, 1990; Williams *et al.*, 1990) and virus exposure (Oldstone, 1988, 1990; Oldstone *et al.*, 1990; Wilberz *et al.*, 1991) have been considered as causing the differences in the incidence of diabetes, their precise effects are still unknown.

III. Clinical and Histopathological Characteristics

NOD mice spontaneously and suddenly develop diabetes without any external prodromes. Polydypsia (>20 ml/day), polyuria (>18 ml/day), severe glycosuria (Testape >1/4%), hyperglycemia (>500 mg/dl), and insulin deficiency (<1.0 μU/ml) are observed in these animals, accompanied by a rapid weight loss (Makino *et al.*, 1980; Fushimi *et al.*, 1980). Ketonuria is usually observed in female mice, and the number of animals exhibiting positive ketonuria increases with time after the onset of diabetes. Spontaneous remission is rarely observed, and these diabetic animals die within 1 to 2 months after the onset of diabetes unless they receive insulin therapy. A clear sex difference is observed with respect to the onset of diabetes. In females, the onset of diabetes is observed at approximately 10 weeks of age, and the number of animals developing overt diabetes increases with age; the cumulative incidence of diabetes by the age of 30 weeks in females is 70–80%. By contrast, in the case of male animals, the onset of diabetes is observed to begin at around 20 weeks of age, and the number of animals affected by the age of 30 weeks is only about 20% (Makino *et al.*, 1980).

Histopathological examination of the pancreas of the NOD mouse reveals characteristic infiltration of mononuclear cells into the pancreatic islets (insulitis). Although insulitis is not observed in NOD mice up to 3 weeks of age, it is observed at high incidences in both female and male animals after 5 weeks of age or older. The cumulative incidence of animals exhibiting insulitis reaches almost 100% by 30 weeks of age. Severe cell infiltration is frequently observed in animals at the age of 9 or 10 weeks, but these mice do not develop overt diabetes. However, many pancreatic islets of diabetic animals show atrophy, and infiltrating cells disappear from the interior of these atrophied pancreatic islets and are only observed in small numbers around the periphery of the islets (Makino *et al.*, 1980). β cells, which secrete insulin, are not observed inside these pancreatic islets. Most of the mononuclear cells infiltrating into the pancreatic islets are Thy-1-positive T cells. Although the majority are CD4-positive cells, a small number of CD8-

positive cells are also observed. MAC1-positive cells and asialo-GM_1-positive NK cells are found in the periphery of pancreatic islets; however, they are not observed inside the pancreatic islets. No notable change in α, δ, and pancreatic polypeptide (PP) cells, consisting of the pancreatic islets, is observed (Fujita *et al.*, 1982). In NOD mice, infiltration of mononuclear cells is also frequently observed in the submandibular and lacrimal glands in females and males, respectively, older than 9 weeks of age.

In 1977, Nakhooda *et al.* (1977) developed the BB rat as an animal model for type I diabetes mellitus. Its clinical and histopathological characteristics are similar to those of the NOD mouse, except for a high incidence of overt diabetes in males as well as in females, and thyroiditis (Wright *et al.*, 1983).

IV. Genetics

A. MHC-Linked Diabetogenic Genes

One of the susceptibility genes is closely linked to the class II major histocompatibility complex of NOD mice, the haplotype of which has been designated $H\text{-}2^{g7}$. The development of diabetes is dependent on homozygosity for the NOD major histocompatibility region in (C3H × NOD) × NOD backcross progeny or F_2 mice (Hattori *et al.*, 1986). This has been confirmed in backcrossing of NOD to other strains, such as NON or C57BL/10 (B10) (Prochazka *et al.*, 1987; Wicker *et al.*, 1987). Moreover, neither insulitis nor diabetes has been observed in the H-2 congenic NOD strains—NOD.B10-H-2 ($H\text{-}2^b$), NOD.B6-H-2 ($H\text{-}2^b$), NOD.NON-H-2 ($H\text{-}2^{nb1}$), and NOD.NCT-H-2 (Wicker *et al.*, 1990; Leiter, 1990; S. Makino, unpublished). The only exception is NOD.CTS-H-2, which shares the class II region with NOD mice and develops diabetes. Interestingly, mild insulitis is observed in F_1 mice crosses between NOD and these congenic strains (Wicker *et al.*, 1990; Leiter, 1990; S. Makino, unpublished). Therefore, the H-2-linked diabetogenic gene(s) of NOD appear to be dominant, with incomplete penetrance, rather than recessive.

The MHC class I of NOD mice is identical to $H\text{-}2K^d$ and $H\text{-}2D^b$. However, the NOD mouse has several unique characteristics in the class II and III region. First of all, the I-A molecule of these mice is serologically and structurally different from those of other laboratory strains (Hattori *et al.*, 1986; Acha-Orbea and McDevitt, 1987). The whole sequence of the NOD Aα chain is identical to that of $A\alpha^d$. Although the second external domain and the transmembrane and intracellular regions of NOD Aβ are also identical to $A\beta^d$, the first external domain of NOD Aβ is unique (Acha-Orbea and McDevitt, 1987). Particularly, NOD has histidine–serine at positions 56 and 57 of the Aβ chain, whereas conserved amino acids, proline–aspartic acid or serine–aspartic acid, are observed in other Aβ chains of $H\text{-}2^{k,b,d,s,u,f,q}$. The A$\beta$ allele of NOD Aβ has been subsequently detected in ICR-derived, diabetes-resistant sister strains, CTS/Shi and ILI/Shi (Ikegami *et al.*, 1988, 1990; Koide and Yoshida, 1990). Similarly, uncharged amino acids such as serine or valine are observed at position 57 in DQβ chains of Caucasian patients with IDDM, whereas β chains of an IDDM-resistant *DQ* haplotype have an aspartic acid at this position (Todd *et al.*, 1987, 1988). On the basis of the computer model of class II molecules, it has been postulated that Asp 57 at the end of the α helix of the β chain, which is a side wall of an antigen-binding groove, may form a salt bridge with Arg 79 at the end of the α helix of the α chain, the other side wall (Brown *et al.*, 1988). Therefore, the substitution of Asp 57 in uncharged amino acids may result in a conformational change of an antigen-binding groove.

A second characteristic of $H\text{-}2^{g7}$ is a lack of *I-E* expression (Hattori *et al.*, 1986). This is probably due to the defect of the promoter region of the Eα gene and is also observed in some other inbred mice, such as C57BL/6 (B6) and C57BL/10 (B10) (Mathis *et al.*, 1983).

In addition to the class II region, the class III region of NOD also have some interesting features. Restriction fragment-length protein (RFLP) analysis has suggested that NOD carries a unique *HSP70* allele, which is mapped between *C4* and *Tnf-α* in the class III region (Gaskins *et al.*, 1990). It is noteworthy that an IFN-γ-inducible "occult" class I-like molecule, which is detected by an anti-$H\text{-}2K^{b,f}$ monoclonal antibody, has been found on islet β cells and macrophages of NOD mice (Leiter *et al.*, 1989). Although it is not known where the gene encoding this molecule is located, it has been suggested that its cross-reactivity with an anti-$H\text{-}2K^{b,f}$ monoclonal antibody may be related to the similarity between the $H\text{-}2^{g7}$ and $H\text{-}2^b$ haplotypes from the *Tnf-α* locus distal to the *Q7/Q9* region (Leiter, 1990; Lund *et al.*, 1990a).

Among the unique characteristics described above, both an absence of I-E expression and the structure of $I\text{-}A^{g7}$ have been demonstrated, using transgenic mice, to contribute to the disease susceptibility (Table I). Nishimoto *et al.* (1987) have backcrossed the Eα-transgenic B6 mouse to NOD. One-fourth of BC 1 progeny have expressed I-E molecules in the background of $H\text{-}2^{g7}$, since the Eα transgenes are not integrated into chromosome 17 of the transgenic mouse. None of these

TABLE I
DEVELOPMENT OF INSULITIS AND DIABETES IN MHC CLASS II-TRANSGENIC NOD MICE

Transgene	Introduction	Insulitis	Diabetes	Ref.
$E\alpha^d$	Backcross[a]	Suppressed	Suppressed	Nishimoto *et al.* (1987); H. Kikutani, unpublished
$E\alpha^k$	Backcross	Suppressed	—	Böhme *et al.* (1990)
Promoter-mutated $E\alpha^{kb}$	Backcross	Not affected	—	Böhme *et al.* (1990)
$E\alpha^d$	Microinjection	Suppressed	Suppressed	Uehira *et al.* (1989); H. Kikutani, unpublished
$E\alpha^d$	Microinjection	Suppressed	Suppressed	Lund *et al.* (1990b)
$A\beta^k$	Microinjection	Not affected	Not affected	Uehira *et al.* (1989)
$A\alpha^k + A\beta^k$	Microinjection	Suppressed	Suppressed	Miyazaki *et al.* (1990)
$A\alpha^k + A\beta^k$	Microinjection	Suppressed	Suppressed	Slattery *et al.* (1990)
$A\alpha^k$ + mutated $A\beta^{kc}$	Microinjection	Suppressed	Suppressed	Miyazaki *et al.* (1990)
Mutated $A\beta^{nodd}$	Microinjection	Suppressed	Suppressed	Lund *et al.* (1990b)

[a] The transgenes were introduced into NOD mice by backcrossing transgenic mice of different strains to NOD mice.
[b] Three mutated $E\alpha^k$ genes (ΔX, ΔY, and *Sma*) carrying deletions in the promoter region, whose expression is limited to particular compartments of the immune system.
[c] The mutated gene encoding $A\beta^k$ with Ser 57 instead of Asp.
[d] The mutated gene encoding $A\beta^{nod}$ with Pro 56 instead of His.

mice have developed insulitis, suggesting that the expression of I-E can prevent insulitis and diabetes. Similarly, Böhme *et al.* (1990) have backcrossed the wild-type $E\alpha^k$ or promoter-mutated $E\alpha^k$-transgenic mice to NOD and have found that the expression of the wild-type $E\alpha^k$ transgene in the NOD background can prevent insulitis. However, insulitis has been observed in BC mice carrying promoter-mutated transgenes and expressing I-E only in certain subsets of class II-expressing immunocompetent cells. The protective effect of I-E has also been confirmed with the generation of the $E\alpha$-transgenic NOD lines by injecting the $E\alpha$ gene directly into NOD embryos (Uehira *et al.*, 1989; Lund *et al.*, 1990b). The same approach with transgenic mice has been taken to assess the diabetogenic feature of I-A^{g7} (Miyazaki *et al.*, 1990; Slattery *et al.*, 1990; Lund *et al.*; 1990b). The expression of I-A^k has significantly reduced incidence of insulitis in $A\alpha^k$ and $A\beta^k$ double-transgenic NOD mice (Miyazaki *et al.*, 1990; Slattery *et al.*, 1990). However, the expression of I-A^k carrying mutated $A\beta^k$ with serine substitution at position 57 has also showed a comparable protective effect (Miyazaki *et al.*, 1990). Lund *et al.* (1990b) have produced the transgenic NOD mice expressing the mutated $A\beta^{nod}$ by substituting the histidine with proline at position 56, and have shown that

the expression of this mutant $A\beta$ has also significantly reduced the incidence of insulitis. These results suggest that His 56 may participate in determining the conformation of $A\beta^{nod}$ responsible for diabetogenesis. However, the preventive effect of $A\alpha^k$ and the mutated $A\beta^k$ carrying for Ser 57 does not necessarily mean that Ser 57 is not a diabetogenic feature. Rather, this suggests that other sequences of $A\beta^{nod}$ or $A\alpha^{nod}$ are also required for disease susceptibility. Therefore, production of transgenic mice carrying the mutated $A\beta^{nod}$ with amino acid substitution at position 57 will be also necessary.

As described above, all the MHC-transgenic experiments indicate that both an absence of I-E and the structure of I-A^{g7} appear to be responsible for the diabetogenic feature of the $H\text{-}2^{g7}$ haplotype. However, this does not necessarily exclude the possibility that other gene(s) of the NOD MHC region may influence the development of insulitis and diabetes. It is noteworthy that a reduced incidence of insulitis has been observed in the congenic NOD.CTS-H-2 strain, which shares the class II but not the class I region with NOD mice (S. Makino, unpublished). Therefore, one must keep in mind the possibility that the combination of unusual or common components encoded in the NOD MHC may form the $H\text{-}2^{g7}$-linked diabetogenic feature.

B. NON-MHC-LINKED GENES

The backcross studies of NOD mice with diabetes-resistant strains have revealed that in addition to an MHC-linked gene or gene complex, three or four additional genes control the development of overt diabetes.

Genetic analysis of [(NOD × NON) F_1 × NOD] first and second backcross generations by Prochazka *et al.* (1987) has also shown that three recessive genes, which have been named *Idd-1*, *Idd-2*, and *Idd-3*, are required for development of diabetes. *Idd-1* is tightly linked to the *H-2K* locus on chromosome 17 and *Idd-2* is localized proximal to but weakly linked to the *Thy-1/Alp-1* cluster on chromosome 9 (Prochazka *et al.*, 1987, 1989). The third gene, *Idd-3*, has been segregated in the second generation of backcross; however, its chromosomal localization is unknown. Wicker *et al.* (1987) have independently analyzed the development of diabetes and insulitis in (NOD × C57BL/10) F_1, F_2, and (F_1 × NOD) backcross generations and have shown that diabetes is controlled by at least three functionally recessive diabetogenic genes, one of which is linked to the NOD MHC. Their results have suggested that one of non-MHC-linked genes may be essential for the development of insulitis and that the MHC-linked gene may modulate the incidence or the severity of insulitis.

Recently, they have determined the chromosomal localization of these non-MHC-linked genes in collaboration with Todd and colleagues (1991; Comall *et al.*, 1991). The congenic B10 *H-2*^{g7} strain has been used as a backcross partner instead of B10 to fix the influence of the MHC-linked *Idd-1* in the backcross progeny. Genetic mapping using microsatellite sequences as chromosomal markers has revealed three genes, *Idd-3*, *Idd-4*, and *Idd-5*. *Idd-3* is located near the marker *D3Ndsl* on chromosome 3, and may be identical to "*Idd-3*" reported by Prochazka *et al.* (1987; E. H. Leiter, personal communication). This affects both insulitis and diabetes and may be the susceptibility gene previously proposed to be essential for the development of insulitis (Wicker *et al.*, 1987). *Idd-4* is located near the marker *D11Ndsl* on chromosome 11. This gene may influence the frequency of insulitis or may control the progression to overt diabetes. *Idd-5* is linked to the interleukin-1 receptor (Il-1r) and the *Lsh/Ity/Bcg* genes of chromosome 1. Interleukin-1 is known as an important cofactor of T cell activation. It has been shown to have a direct toxic effect on pancreatic β cells (Mandrup-Poulsen *et al.*, 1986). Paradoxically, *in vivo* administration of IL-1 prevents diabetes in NOD mice (Jacob *et al.*, 1990). *Lsh/Ity/Bcg* determines susceptibility to *Leishmania donovani* (*Lsh*), *Salmonella typhimurium* (*Ity*), and *Mycobacterium bovis* (bacillus calmette–Guérin, *Bcg*), which infect macrophages, and influences the capacity of macrophages to restrict intracellular proliferation of these microorganisms (Schurr *et al.*, 1991; Blackwell *et al.*, 1991; Maro *et al.*, 1991). Therefore, both can be a candidate for *Idd-5*. Interestingly, a gene similar to *Idd-5* has been identified as controlling periinsulitis, which is the earliest pathological feature of diabetes in NOD mice (Garchon *et al.*, 1991). It is thus likely that these diabetogenic genes play roles at different stages, between the initiation of autoimmunity and the progression to overt diabetes. *Idd-2* on chromosome 9, which may influence diabetes in a (NOD × NON) F_1 × NOD backcross, was not seen in a (NOD × B10.*H-2*^{g7}) × NOD backcross. These diabetogenic genes, including *Idd-1*, *Idd-3*, *Idd-4*, and *Idd-5*, are not strictly recessive. Diabetes occurred in BC 1 progeny that were heterozygous at *Idd-1*, *Idd-3*, *Idd-4*, or *Idd-5*. Therefore, these non-MHC-linked genes as well as MHC-linked genes seem to be dominant with incomplete penetrance.

V. Autoimmunity

A. T Cell-Dependent Autoimmunity

The immunohistological studies of pancreatic islets have revealed that T lymphocytes as well as B lymphocytes, monocytes, and NK cells

infiltrate into islets, although there are conflicting data among these reports concerning the ratio of each lymphocyte subset (Kanazawa *et al.*, 1984; Miyazaki *et al.*, 1985; Signore *et al.*, 1989). However, it is noteworthy that CD4 T lymphocytes are predominantly infiltrating compared to CD8 T lymphocytes, and that 30% of T lymphocytes express interleukin-2 receptors (Signore *et al.*, 1989), suggesting the active participation of T lymphocytes, particularly CD4$^+$ cells in insulitis. Direct and indirect evidence of T lymphocyte-dependent autoimmune etiology of insulitis and diabetes in NOD mice stems from early studies on neonatal thymectomized or athymic NOD mice and administration of immunosuppressive drugs or anti-T cell reagents to NOD mice (Ogawa *et al.*, 1985; Makino *et al.*, 1986; Harada and Makino, 1986; Mori *et al.*, 1986). Neonatal thymectomy significantly reduces the incidence of insulitis and diabetes (Ogawa *et al.*, 1985). More directly, neither insulitis nor diabetes is observed in athymic nude NOD mice (Makino *et al.*, 1986). Suppression of the disease onset is also observed in mice injected with monoclonal anti-Thy-1.2 antibody or cyclosporin A, which is known to impair T lymphocyte function (Harada and Makino, 1986; Mori *et al.*, 1986). To date, a number of studies in which NOD mice were treated with antibodies to T lymphocyte surface antigens or molecules involved in T cell recognition have been reported, as shown in Table II. Most antibodies can suppress the

TABLE II
Effects of Anti-Lymphocyte Antibodies

Antibody	Disease	Effect	Ref.
Anti-Thy-1.2	Spontaneous DM[a]	Suppression	Harada and Makino (1986)
Antithymocyte serum	Spontaneous DM	Suppression	Harada and Makino (1986)
Anti-CD4	Spontaneous DM	Suppression	Koike *et al.* (1987)
Anti-CD4	Spontaneous DM	Suppression	Shizuru *et al.* (1988)
Anti-CD4	CY-induced DM[b]	Suppression	Charlton *et al.* (1988)
Anti-CD4	Transferred DM	Suppression	Varey *et al.* (1991)
Anti-CD8	Cy-induced DM	Suppression	Charlton *et al.* (1988)
Anti-CD8	Transferred DM[c]	Suppression	Hutchings *et al.* (1990a); Varey *et al.* (1991)
Anti-CD3	Spontaneous DM	Suppression	Hayward and Shreiber (1989)
Anti-α/βTCR	Spontaneous and CY induced	Suppression	Sempé *et al.* (1991)
Anti-IL-2 receptor	Spontaneous DM	Suppression	Kelley *et al.* (1988)
Anti-I-A	Spontaneous DM	Suppression	Boitard *et al.* (1988)

[a] Spontaneously developing diabetes mellitus.
[b] Cyclophosphamide-induced diabetes mellitus.
[c] Diabetes mellitus transferred by spleen cells from diabetic mice.

development of insulitis or diabetes, although their effects vary depending on the dose or the treatment period.

B. T Lymphocyte Subsets Involved in Insulitis and Overt Diabetes

Elimination of $CD4^+$ T lymphocytes by *in vivo* administration of a monoclonal anti-CD4 antibody can suppress both insulitis and diabetes in NOD mice (Koike *et al.*, 1987; Shizuru *et al.*, 1988). This suppressive effect in mice is observed following long-term treatment (i.e., 100 μg/week) but not following short-term treatment (i.e., 600 μg in the first 3 days) (Shizuru *et al.*, 1988). An anti-CD4 antibody can also block a cyclophosphamide-promoted progression to diabetes in NOD mice that already display insulitis (Charlton and Mandel, 1988). These results suggest that $CD4^+$ T lymphocytes may play an essential role not only in an initiation of insulitis but also in progression to diabetes.

Evidence for involvement of $CD8^+$ T lymphocytes has been also obtained from several cell transfer experiments. Severe insulitis and diabetes can be successfully transferred to young irradiated or neonatal NOD mice using spleen cells or T lymphocytes from diabetic mice (Wicker *et al.*, 1986; Bendelac *et al.*, 1987). Depletion of either $CD4^+$ or $CD8^+$ T lymphocytes from donor cells abrogates the transfer of diabetes (Bendelac *et al.*, 1987; Miller *et al.*, 1988). Therefore, both cell types are necessary for transfer of diabetes. In acute transfer of diabetes to neonatal NOD mice, $CD4^+$ cells are predominantly seen at the early phase of periinsular infiltration, whereas the proportion of $CD8^+$ cells in infiltrating T cells substantially increases at the time of destruction of insulin-producing cells (Bedossa *et al.*, 1989). $CD4^+$ cells alone but not $CD8^+$ cells can induce insulitis when transferred into irradiated T lymphocyte-depleted (Hanafusa *et al.*, 1988), athymic nude (Harada *et al.*, 1990a) or neonatal NOD mice (Thivolet *et al.*, 1991). Immunohistologic studies have shown that islet infiltration of recipients of $CD4^+$ cells alone is mild and consists predominantly of $CD4^+$ cells, whereas islets of recipients of both subsets are heavily infiltrated with both $CD4^+$ and $CD8^+$ cells. These findings suggest that $CD4^+$ cells may be involved in the initiation of insulitis and homing of $CD8^+$ cells to islets and that $CD8^+$ cells may promote islet destruction and overt diabetes. This possibility may fit the concept of T–T interaction, where MHC class II-restricted $CD4^+$ T cells help class I-restricted cytotoxic $CD8^+$ cells. However, administration of anti-CD8 antibodies after transfer of diabetic spleen cells has been shown to prevent intraislet infiltration of $CD4^+$ cells and macrophages as well as overt diabetes in irradiated recipients (Hutchings *et al.*, 1990a). This can also be interpreted as a role played by $CD8^+$ cells in facilitating the influx of $CD4^+$ cells and macrophages to an intraislet location (Hutchings *et al.*, 1990a; Varey *et al.*, 1991).

In the transfer of polyclonal T lymphocytes, both subsets are necessary for induction of overt diabetes; however, a requirement for $CD4^+$ and $CD8^+$ subsets is still controversial in assessing disease transfer by T cell clones. Reich *et al.* (1989) have established $CD4^+$ and $CD8^+$ T cell clones both of which are islet specific and NOD MHC restricted. Neither the $CD4^+$ nor the $CD8^+$ clone alone can induce severe insulitis when injected into irradiated young NOD or (NOD × BALB/c) F_1 mice; however, a mixture of $CD4^+$ and $CD8^+$ clones causes intense insulitis as well as glycosuria on occasion. Haskins *et al.* (1989) have established several $CD4^+$ T cell clones that are reactive to islet cells in the context of the NOD MHC. In contrast to the report by Reich *et al.* (1989), some of these clones can induce diabetes when transferred into unirradiated young NOD mice (2 to 3 weeks of age) (Haskins and McDuffie, 1990). The latter shows that primed $CD4^+$ T cells can accelerate diabetes in the absence of primed $CD8^+$ cells. It is conceivable that if islet-reactive $CD4^+$ T cells are aggressive enough, they can initiate the disease process and may recruit T cells of host origin even in young mice.

It has been proposed that stimulation of autoreactive $CD4^+$ T lymphocytes by aberrantly expressed class II MHC antigens may trigger endocrine autoimmunity including IDDM (Bottazzo *et al.*, 1983; 1985; Hanafusa *et al.*, 1987; Formby and Miller, 1990). However, most of the immunohistological and flow cytometric analyses have demonstrated that the class II MHC antigens are expressed on bone-marrow-derived cells but not on β cells on NOD pancreatic islets (Signore *et al.*, 1989; McInerney *et al.*, 1991). It is worth noting that the class I MHC antigens increase with age on β cells of NOD islets (McInerney *et al.*, 1991). Taking these observations into consideration together with the requirement for $CD4^+$ and $CD8^+$ T lymphocytes for IDDM induction, it is reasonably assumed that autoreactive $CD4^+$ T lymphocytes, which recognize and respond to self antigens presented by class II-expressing antigen-presenting cells such as macrophages and B lymphocytes, help class I-restricted cytotoxic $CD8^+$ T lymphocytes, which in turn destroy pancreatic β cells. Terada *et al.* (1988) have transplanted pancreatic islets from newborn NOD, BALB/c, B10, or CBA mice into diabetic NOD mice that were treated with cyclosporin A to prevent allogeneic rejection. Pancreatic islets from NOD, B10, and BALB/c mice, but not from CBA mice, have been rejected by cyclosporin A-treated diabetic NOD mice. NOD mice share *H-2-K*^{d} and *H-2-D*^{b}

with BALB/c and B10, respectively. This suggests that the class I-restricted effector mechanism may be involved in autoimmune β cell destruction in NOD mice. Nagata *et al.* (1989) have reported that spleen cells from NOD mice, as shown by activity in a ^{51}Cr release assay, lyse islet cells from NOD, BALB/c, DBA/2, and B10.GD mice, which share *H-2K*^{d}. A depletion experiment using anti-T lymphocytes reagents has shown that such cytotoxic activity is mediated by CD8$^+$ T lymphocytes. However, unlike an *in vivo* study by Terada *et al.* (1988), they have failed to demonstrate *H-2D*-restricted cytotoxicity against islet cells in their *in vitro* system.

Non-MHC-restricted cytotoxicity also has been suggested for β cell destruction. Wang *et al.* (1987) have transplanted pancreatic islets and other control tissues from BALB/c, CBA, and SJL mice into diabetic NOD mice after *in vitro* culture to prevent allogeneic graft rejection. In this system, islets from *H-2*-incompatible CBA and SJL mice, as well as from *H-2K*-compatible BALB/c mice, have been rejected, whereas other control tissues have been retained. Furthermore, *in vivo* treatment of recipient mice with anti-CD4 antibody has abrogated islet rejection. This result suggests that the destruction of islets may result rather from CD4$^+$ T lymphocyte-mediated nonspecific inflammatory process, in which T lymphocyte-derived cytokines, cytokine-activated effector cells such as LAK and NK cells and macrophages, macrophage-derived cytokines, and free radicals may play major roles. Timsit *et al.* (1988) have shown that spleen cells from diabetic NOD mice inhibit the insulin release from islet cells of NOD and DBA/2 mice and even Wistar rats, and that these inhibitory cells are CD8$^+$, suggesting the involvement of nonspecific effector cells. In fact, the possibility has also been suggested that NK cells may be responsible for islet cytotoxicity in IDDM of BB rats.

C. The Repertoire of Diabetogenic T Lymphocytes

In other organ-specific autoimmune models, particularly experimental autoimmune encephalomyelitis (EAE), the T cell receptor (TCR) usage of autoreactive T lymphocytes has been extensively studied. EAE can be induced in mice and rats by immunizing with myelin basic proteins (MBPs) (Zamvil and Steinman, 1990). It has been demonstrated that most PL- or B10.PL-derived T cell clones, which are reactive to the NH_2-terminal peptide of MBP, utilize V_β8.2 or 13 and V_α2 or 4 (Urban *et al.*, 1988; Acha-Orbea *et al.*, 1988; Zamuil *et al.*, 1988). These indicate a restricted usage of TCRs in autoreactive T cells of EAE. Similar evidence for a limited heterogeneity of autoreactive T cells has also been obtained from studies of MBP-reactive T cells from

rats or other strains of mice. However, it is still controversial whether these results, obtained from experimentally induced autoimmune models can be applicable to spontaneously developing autoimmune diseases such as type I diabetes, multiple sclerosis, and rheumatoid arthritis. In this respect, it is very interesting to consider TCR usage of autoreactive T cells in autoimmune insulitis in NOD mice.

As described before, various kinds of cells, including CD4$^+$ and CD8$^+$ T lymphocytes, B lymphocytes, monocytes, and NK cells, infiltrate into islets even in the early phase of insulitis in NOD mice (Kanazawa *et al.*, 1984; Miyazaki *et al.*, 1985; Signore *et al.*, 1989). This may imply polyclonal lymphocyte infiltrations. In fact, the analysis using anti-V_β reagents has shown that the V_β usage of islet-infiltrating T lymphocytes is very similar to that of lymph node T lymphocytes (Nakano *et al.*, 1991). Maeda *et al.* (1991) have analyzed the V_β gene expression of infiltrating T lymphocytes using the polymerase chain reaction and have obtained a similar result except for predominant V_β11 expression in infiltrating T lymphocytes from young (5-week-old) NOD mice. These results have demonstrated that TCR gene usage of infiltrating T lymphocytes is rather heterogeneous. However, this may not reflect TCR usage of islet-reactive T lymphocytes, which are actually involved in the initiation of autoimmune insulitis.

Bacelj *et al.* (1989) have shown that *in vivo* treatment with anti-V_β8 monoclonal antibody can prevent cyclophosphamide-induced diabetes in NOD mice, suggesting that V_β8 T lymphocytes may play an essential role in the destruction of pancreatic islets. Reich *et al.* (1989) have reported that insulitis and diabetes can be transferred with a mixture of NOD islet-reactive CD4 and CD8 T cell clones, both of which express V_β5. These results, together with the fact that diabetes can be prevented by I-E expression in transgenic NOD mice (Nishimoto *et al.*, 1987), indicate that involvement of V_β5 T cells seems to be an essential requirement for the development of diabetes. Recently, Fathman and colleagues have studied the TCR V_β requirement in autoimmune diabetes by crossing NOD mice with SWR mice (Livingstone *et al.*, 1991; Shizuru *et al.*, 1991). NOD has the TCR $V\beta^b$ haplotype, encoding ~20–25 functional V_β gene elements, whereas SWR has the V_β^a haplotype and carries the deletion of ~10 V_β genes segments, including the V_β5, V_β8, and V_β11 gene families. They have observed similar incidences of insulitis and diabetes in both $V_\beta^{a/b}$ and $V_\beta^{a/a}$ mice homozygous for *H-2*^{g7} selected from [(NOD × SWR) × NOD] backcross progenies. One diabetic *H-2*$^{g7/g7}$ and $V_\beta^{a/b}$ female has been backcrossed to NOD and the resulting $V_\beta^{a/b}$ second backcross progenies, which retain all diabetogenic genes, have

been intercrossed. The development of insulitis and overt diabetes has been observed in $V_\beta^{a/a}$ as well as $V_\beta^{a/b}$ and $V_\beta^{b/b}$ mice. These results have demonstrated that V_β gene segments deleted in the V_β^a haplotype, including $V_\beta 5$ and $V_\beta 8$ gene families, are not essential requirements for the disease development. In fact, TCRs of T cell clones established by Reich *et al.* (1989) have recently been found to be encoded by $V_\beta 1$ genes but not by $V_\beta 5$ genes.

There have been established a number of islet-reactive T cell clones or hybrids (Haskins *et al.*, 1989; Nakano *et al.*, 1991; Reich *et al.*, 1989; Prud'homme *et al.*, 1990). TCR gene usage of some of these clones has been analyzed. Nakano *et al.* (1991) have established five $CD4^+$ T cell clones, all of which are reactive to islet cells in the context of the NOD MHC and can induce insulitis when transferred into disease-free I-E-transgenic NOD mice (Uehira *et al.*, 1989). TCR sequences of these T cell clones have been cloned and determined by anchored polymerase chain reactions. Candéias *et al.* (1991) have also cloned and sequenced TCRs from $CD4^+$ islet-reactive T cell clones (Haskins *et al.*, 1989). The results from both groups are shown in Table III. Each clone utilizes a distinct combination of V_α, B_β, J_α, and J_β gene segments and no particular combination has been shared by more than two clones. Prud'homme *et al.* (1990) have also analyzed TCR V_β expression of several NOD-derived T cell hybrids that are reactive to islet cells, and found that several V_β elements are represented with no obvious dominant type in these hybrids, although some of them are not yet cloned. All of these results have demonstrated that TCR gene usage is not restricted,

TABLE III
T CELL RECEPTOR GENE USAGE OF NOD ISLET-REACTIVE T LYMPHOCYTE CLONES[a]

Clone	V_α	J_α	V_β	J_β
4-1-L.6[b]	$V_\alpha 13$	$J_\alpha TT11$	$V_\beta 15$	$J_\beta 2.7$
4-1-K.1[b]	$V_\alpha 6.1$	$J_\alpha TA27$	$V_\beta 8.2$	$J_\beta 2.7$
4-1-G.4[b]	$V_\alpha 2.2$	$J_\alpha E1$	$V_\beta 16$	$J_\beta 2.2$
4-1-E.2[b]	$V_\alpha 1.1$	$J_\alpha TA80$	$V_\beta 12$	$J_\beta 2.5$
7-10-D.3[b]	$V_\alpha 1.1$	$J_\alpha D3$	$V_\beta 15$	$J_\beta 1.3$
BDC2.5[c]	$V_\alpha BDC2.5$	$J_\alpha TA28$	$V_\beta 4$	$J_\beta 1.2$
BDC4.12[c]	$V_\alpha BDC4.12$	$J_\alpha TA80$	$V_\beta 19$	$J_\beta 2.6$
BDC5.2[c]	$V_\alpha 12$	$J_\alpha TA28$	$V_\beta 6$	$J_\beta 2.1$
BDC6.9[c]	$V_\alpha 13.1$	$J_\alpha TA72$	$V_\beta 4$	$J_\beta 2.3$

[a] Adapted from Nakano *et al.* (1991) and Candéias *et al.* (1991).
[b] Clones established by Nakano *et al.* (1991).
[c] Clones established by Haskins *et al.* (1989).

even in T cell clones, some of which can actually transfer disease. This is in great contrast to the limit heterogeneity in TCR V_α and V_β gene usage in MBP-reactive T cells in EAE, and raises several possibilities. First, one or a few diabetogenic T cell clones may primarily initiate autoimmunity and trigger a chain reaction that results in secondary and polyclonal autoaggressive T cells, which the T cell clones described above may represent. Second, putative diabetogenic self antigens in NOD mice may elicit relatively heterogeneous T cells in the context of $I\text{-}A^{g7}$. Third, autoantigens in pancreatic islets may be isolated from direct contact with immunocompetent cells so that T cells potentially reactive to such antigens are not tolerated but are just unprimed. If in some unknown way, pancreatic islets are damaged, any proteins specific to pancreatic islets may elicit corresponding T cells. At the moment, we do not know which of these or any other possibilities is the case. The characterization of autoantigens recognized by islet-reactive T lymphocytes may provide a clue toward understanding the generation of autoreactive T lymphocytes.

D. A Suppressor Mechanism Controlling the Development of Diabetes

The incidence of diabetes is considerably low in young male NOD mice in most colonies and in female mice in one colony (Makino *et al.*, 1980; Charlton *et al.*, 1988). Cyclophosphamide treatment can accelerate the development of diabetes in such disease-resistant mice (Harada and Makino, 1984; Charlton *et al.*, 1989). Transfer of T lymphocytes from diabetic mice can induce diabetes in irradiated young mice but not in unirradiated young mice (Wicker *et al.*, 1986). These observations suggest that cyclophosphamide treatment and irradiation may abrogate a protective mechanism in disease-resistant mice. Both regimes have been used to inactivate "suppressor T lymphocytes." Several transfer experiments have suggested that a certain subpopulation of T lymphocytes may play a protective role in the control of diabetes development. Yasunami and Back (1988) and Charlton *et al.* (1989) have shown that T cells from cyclophosphamide-treated NOD mice are capable of transferring the disease to irradiated recipients and suggested that the diabetogenic effect of cyclophosphamide is not mediated by direct toxicity on β cells, but is mediated by abrogation of a mechanism that protects mice from the disease. Although transfer of spleen cells from diabetic mice can induce diabetes in irradiated recipients, reconstitution of recipients with spleen cells from prediabetic mice 24 hours or 6 days prior to transfer can prevent disease induction (Boitard *et al.*, 1989; Hutchings and Cooke, 1990). Cell depletion

experiments have shown that the protective effect is mediated by $CD4^+$ T cells Boitard *et al.*, 1989; Hutchings and Cooke, 1990). Furthermore, thymectomy at weaning can abrogate the suppressive capacity of spleen cells from female mice but not from male mice, to confer protection against diabetes transfer (Boitard *et al.*, 1989; Dardenne *et al.*, 1989). A similar involvement of suppressor T lymphocytes has been proposed in thymectomy-induced organ-specific autoimmunity (Sakaguchi *et al.*, 1982a,b). Boitard *et al.* (1988) have shown that *in vivo* treatment by anti-class II monoclonal antibody can prevent diabetes and that this disease protection can be transferred by splenic T cells, suggesting that anti-class II antibody induces suppressor T lymphocytes. However, the mechanism of generation of suppressor cells by anti-class II treatment is unknown. Reich *et al.* (1989) have established an autoreactive T lymphocyte clone specific to $I\text{-}A^{g7}$ from islets of newly diabetic NOD mice. *In vivo* administration of this clone to prediabetic mice has almost completely aborted the development of diabetes. This treatment can also prevent insulitis. It remains to be clarified how this T lymphocyte clone is relevant to suppressor cell populations in prediabetic mice or anti-class II-treated mice.

At present, the existence and definition of the suppressor T lymphocyte are controversial. Cohen and colleagues have proposed that resistance to EAE induced by T cell vaccination is mediated by two types of suppressor T lymphocytes (Lider *et al.*, 1988; Lohse *et al.*, 1989). First are the anti-idiotypic suppressor T lymphocytes, which recognize specific anti-MBP receptors (the idiotype) of the autoimmune T lymphocytes (Lider *et al.*, 1988). Second, there are antiergotypic T lymphocytes, which recognize and respond to the state of activation of other T lymphocytes (Lohse *et al.*, 1989). The former may belong to the "classical suppressor T lymphocytes," which are antigen or idiotype specific. The suppressor cell population observed in prediabetic mice may be antiergotypic T lymphocytes. It is noteworthy that NOD mice have a defective activation of suppressor T lymphocytes that are generated in syngeneic mixed lymphocyte reactions (MLRs) and suppress both syngeneic and allogeneic MLRs, and that IL-1 partially restores activity of such suppressor cells (Serreze and Leiter, 1988). Furthermore, as described later, *in vivo* administration of certain cytokines, including IL-1 can prevent diabetes in NOD mice (Jacob *et al.*, 1990). Therefore, such nonspecific suppressors may barely protect mice from the disease at the prediabetic stage. We must also consider other possible mechanisms of protective control of diabetes in addition to an involvement of suppressor T lymphocytes. Interestingly, suppressor cells detected in prediabetic or anti-class II-treated mice cannot

prevent an initiation of autoimmune insulitis but can effectively inhibit promotion from insulitis to diabetes. The latter probably involves more complex immunological and inflammatory phenomena, including the dynamics of a cytokine and interleukin network and a change in the T lymphocyte repertoire. Recently, two subsets of $CD4^+$ helper T lymphocytes, T_{H1} and T_{H2}, have been demonstrated to produce distinct sets of cytokines (Mosmann and Coffman, 1989). An interaction between both subsets has been also demonstrated. These results suggest the heterogeneity of cytokine production by T lymphocytes. Therefore, a change in the proportion of each T lymphocyte subset may influence a progression from insulitis to diabetes. Alternatively, the T lymphocyte repertoire may differ in the prediabetic stage and in the diabetic stage. Thymectomy or cyclosporin A-induced organ-specific autoimmunity has been suggested to involve the fixation of an immature T lymphocyte repertoire, including autoreactivities (Gao *et al.*, 1988; Jenkins *et al.*, 1988; Smith *et al.*, 1989). Transfer of the T lymphocyte repertoire of prediabetic mice may directly or indirectly influence promotion to diabetes.

E. Involvement of Macrophages and B Lymphocytes

If autoimmune insulitis and diabetes in NOD mice are T lymphocyte dependent, the involvement of macrophages is expected from their role in T lymphocyte activation. Administration of silica, which is known to kill macrophages, has been shown to prevent insulitis and diabetes in cyclophosphamide-treated NOD mice (Charlton *et al.*, 1988; Lee *et al.*, 1988) and lymphocyte infiltration into islet grafts (Ihm and Yoon, 1990). Hutchings *et al.* (1990b) have injected the monoclonal antibody 5C6, specific to the myelomonocytic adhesion-promoting type 3 complement receptor (CR3), into irradiated NOD mice that received spleen cells from diabetic mice. This treatment can prevent intraislet infiltration by T lymphocytes and macrophages and can inhibit transfer of diabetes. These results indicate that macrophages also play a role in recruitment of T lymphocytes into islets at an initial stage of insulitis. There are several possible mechanisms by which macrophages might exert an initial role in the immune response against pancreatic islet cells. Macrophages present β cell antigens to class II-restricted autoreactive T lymphocytes. Various kinds of cytokines, including interleukin-1, tumor necrosis factor-α, tumor growth factor-β, and interleukin-6, are produced by macrophages. TNF-α and lymphocyte-derived interferon-γ up-regulate MHC class I and class II antigens and ICAM-1 on pancreatic islet cells, which probably help in the recognition of β cell antigens by T lymphocytes (Campbell *et al.*,

1985; 1988; 1989; Pujol-Borrell *et al.*, 1987). IL-1 and TNF-α may also induce VCAM-1 on vascular endothelial cells, resulting in promoting lymphocyte homing (Osborn *et al.*, 1989). A direct effect on β cells by macrophages has also been suggested. IL-1 alone or in combination with TNF and IFN-γ shows a biphasic effect on glucose-stimulated insulin release of the islets: an inhibition of insulin release at higher concentrations and an increase of insulin release at lower concentrations (Mandrup-Poulsen *et al.*, 1986; Comens *et al.*, 1987; Campbell *et al.*, 1988; Pukel *et al.*, 1988). Macrophages may also destroy β cells through the liberation of free radicals.

The involvement of humoral immunity in disease development in NOD mice is not clear. In humans, autoantibodies to islet cell components, islet cell autoantibodies (ICA), islet cell surface autoantibodies (ICSA), and insulin autoantibodies (IAA) have been detected at an early preclinical stage of IDDM and are suggested to be useful diagnostic markers (Gorsuch *et al.*, 1981; Palmer *et al.*, 1983; Soeldner *et al.*, 1985; Srikanta *et al.*, 1985; Atkinson *et al.*, 1986; Vardi *et al.*, 1987). These antibodies have also been found in sera from NOD mice (Kanazawa *et al.*, 1984; Yokono *et al.*, 1984; Postesilli *et al.*, 1987; Serreze *et al.*, 1988; Michel *et al.*, 1989; Ziegler *et al.*, 1989; Reddy *et al.*, 1990). Furthermore, sera from prediabetic and newly diabetic NOD mice have been shown to react with 64- and 52-kDa β cell antigens (Atkinson and Maclaren, 1988; Karounos and Thomas, 1990). The 64-kDa antibody is observed in rat and human IDDM (Baekkeskov *et al.*, 1984; 1987) and has recently been shown to react with glutamic acid decarboxylase (GAD) (Baekkeskov *et al.*, 1990). However, it is not clear whether these autoantibodies have an initial role in autoimmune insulitis and diabetes. The presence of the above-mentioned antibodies may rather be secondary to the autoimmune destruction of pancreatic islets.

In addition to autoantibodies specific to islet components, NOD mice display humoral autoimmune features observed in lupus-prone mice, including production of antilymphocyte antibodies and hyperglobulinemia, but not anti-DNA antibodies (Kanazawa *et al.*, 1984; Lehuen *et al.*, 1990a;b). Lehuen *et al.* (1990b) have shown that these humoral anomalies are dissociated from cell-mediated autoimmune features and the disease in (NOD × B6) F_1 and (NOD × B6) × NOD backcross mice and have suggested that NOD mice, like other autoimmune strains, suffer from a genetically inherited defect of B lymphocyte regulation, resulting in the hyperproduction of natural autoantibodies.

Watanabe *et al.* (1991) have shown monoclonal B lymphocyte expansion in the thymus of NOD but not I-E-transgenic NOD mice, which

are disease-free animals. However, it remains to be determined whether such B lymphocytes in the thymus are related to natural thymocytotoxic antibodies or autoantibodies to islet components.

Bendelac *et al.* (1988) have transferred purified T lymphocytes from diabetic donors into newborns treated either with a rat antimouse μ monoclonal antibody or with a control antibody, and found induction of diabetes in B lymphocyte-suppressed mice as well as in control mice, suggesting that B cell autoreactivity is a secondary phenomenon that is unimportant during the effector phase of diabetes in NOD mice. However, these observations are not conclusive since there are still residual B lymphocytes in anti-μ-treated mice. Recently, NOD SCID mice have been produced (E. Leiter, personal communication; H. Kikutani, unpublished). These mice will be ideal recipients for transfer of diabetes to determine the role of B lymphocytes in the autoimmune process in NOD mice.

F. Autoantigens

As described previously, several autoantibodies to islet cell components have been detected in sera of prediabetic and diabetic NOD mice. However, little is known about antigens recognized by islet-reactive T cells in these mice. A number of islet-reactive T lymphocyte clones and lines have been established from spleen cells or islet-infiltrating cells of NOD mice (Haskins *et al.*, 1989; Reich *et al.*, 1989; Nakano *et al.*, 1991). Antigens recognized by these T lymphocyte clones have not yet been identified. Haskins *et al.* (1989) have established several islet-reactive clones that react with NOD islet cells in the context of H-2^{g7}. Strain or species specificity analyses of target antigens have suggested that these T lymphocyte clones react with distinct antigenic determinants. Heterogeneity of TCR usage of islet-reactive clones also suggests that multiple antigenic determinants of islet cells are recognized by these T lymphocyte clones (Nakano *et al.*, 1991; Candéias *et al.*, 1991). Elias *et al.* (1990) have reported the appearance of T lymphocytes reactive to the 65-kDa heat-shock protein (HSP) of *Mycobacterium tuberculosis* preceding the onset of insulitis in NOD mice, and subsequently HSP65 cross-reactive antigens become detectable, followed some weeks later by anti-HSP65 and antiinsulin antibodies. HSP65-reactive T lymphocyte lines were able to induce insulitis and diabetes in young NOD mice. Furthermore, vaccination with a T cell epitope of the human 65-kDa HSP protected NOD mice from diabetes (Elias *et al.*, 1991). These findings suggest that a cross-reactive antigen with a 65-kDa HSP of *M. tuberculosis* is one of the target antigens of diabetogenic T lymphocytes,

although it is not known whether pancreatic β cells express such antigens. At present, there is no evidence that islet-reactive T lymphocyte clones (Haskins *et al.*, 1989; Nakano *et al.*, 1991) established from NOD mice react with HSP65 (K. Haskins, personal communication; N. Nakano and H. Kikutani, unpublished).

VI. Intervention of Autoimmune Diabetes

A number of attempts have been made to prevent diabetes in NOD mice, including treatment with antibodies to T lymphocytes or other immunocompetent cells, which were described earlier. Various types of immunomodulation have been found effective in the prevention of diabetes and may be instructive for understanding the autoimmune mechanism and applicable to therapy of human IDDM.

A. Immunopotentiators

Toyota *et al.* (1986) have shown that treatment by a streptococcal preparation (OK-432) suppresses insulitis and inhibits diabetes. Spleen cells of OK-432-treated mice have failed to suppress the transfer of diabetes when given spleen cells from diabetic mice (Shintani *et al.*, 1990). Furthermore, cyclophosphamide, which has been suggested to eliminate suppressor T lymphocytes, cannot induce diabetes in NOD mice injected with OK-432. OK-432 is known to be a strong immunopotentiator that induces various cytokines, including IFN-γ (Saito *et al.*, 1982), IL-1, IL-2 (Ichimura *et al.*, 1985), and TNF (Yamamoto *et al.*, 1986). Therefore, suppressive effects of OK-432 may be due to induction of cytokine production but not to induction of suppressor T lymphocytes.

A single injection of live bacillus Calmette–Guérin (BCG) into young NOD mice significantly reduces the incidence of insulitis and overt diabetes (Harada *et al.*, 1990b). BCG-induced suppression of the disease can be transferred with plastic-adherent cells (possibly macrophages) from BCG-treated mice (Harada *et al.*, 1990b). Sadelain *et al.* (1990) have shown that a single injection of complete Freund's adjuvant (CFA) has a long-lasting preventive effect on overt diabetes. Both reports have suggested that BCG and CFA may exert their protective effect through macrophage and Thy-1-negative and nylon wool-nonadherent natural suppressor cells, respectively (Harada *et al.*, 1990b; Sadelain *et al.*, 1990). In addition, both are strong inducers of cytokines. Thus, it is also possible that certain cytokines induced by these substances may influence the development of diabetes.

B. Cytokines

TNF-α and IL-1 have been shown to inhibit glucose-induced insulin release by pancreatic β cells *in vitro* and have been suggested to be responsible for β cell destruction (Mandrup-Poulsen *et al.*, 1986; Pukel *et al.*, 1988). In contrast to their *in vitro* effect, *in vivo* administration of human and mouse TNF-α causes a significant suppression of insulitis and diabetes in NOD mice (Satoh *et al.*, 1989; Jacob *et al.*, 1990). Human IL-1α can also effectively prevent the disease (Jacob *et al.*, 1990). TNF-α has been shown to prevent lupus nephritis of (NZB × NZW) F_1 mice, which have low TNF levels but high IL-1 levels. Interestingly, production of both TNF-α and IL-1 in NOD mice is significantly reduced compared to other strains. In addition to TNF-α and IL-1, both IL-2 and poly (I:C), which is an inducer of IFN-α and IFN-β, have been shown to be effective in preventing overt diabetes (Serreze *et al.*, 1989). Serreze and Leiter (1988) have shown that NOD mice have a defective activation of suppressor T lymphocytes, which emerge from a syngeneic mixed lymphocyte reaction (SMLR) and suppress SMLRs and allogeneic MLRs and that addition of IL-1 to SMLRs of NOD can partially restore an emergence of such suppressor T lymphocytes. It is unclear that dysfunction of such suppressor T lymphocytes is responsible for the development of diabetes. Taken together, these observations indicate that the protective features of these cytokines involve the modulation of the systemic immune function rather than their direct effect on pancreatic islet cells or infiltrating cells.

C. Immunosuppressants

Immunosuppressive therapy with cyclosporin A has been shown to be effective for patients with IDDM of recent onset (Stiller *et al.*, 1984). A low-dose treatment of NOD mice with cyclosporin A (15 mg/ml every other day) can prevent both insulitis and diabetes (Mori *et al.*, 1986). Recently, a similar but stronger immunosuppressant, FK-506, has been shown to have a preventive effect on insulitis and diabetes (Miyagawa *et al.*, 1990). Cyclosporin A, however, fails to inhibit disease recurrence in the islet graft transplanted to actively diabetic NOD mice (Wang *et al.*, 1988), although it is effective when administered before the onset of the disease. This is in concordance with observations showing that cyclosporin A and FK-506 suppress the early sequence of T lymphocyte activation through the inhibition of the transcription of lymphokines and antigen–signal transduction (Granelli-Piperno *et al.*, 1984; Hodgkin *et al.*, 1987).

D. Virus Infection

Oldstone (1988) has shown that NOD mice infected with a lymphotropic variant of lymphocyte choriomeningitis virus (LCMV) at birth or 6 weeks of age do not become diabetic. Lymphocytes from uninfected NOD mice but not from infected NOD mice can transfer diabetes, suggesting that an interaction between the virus and the lymphocytes is pivotal in aborting diabetes. Lymphotropic LCMV preferentially infects a subset of CD4 T lymphocytes. However, LCMV-infected NOD mice have generated good responses to a variety of non-LMCV antigens, although these responses are somewhat less than those observed in uninfected mice (Oldstone, 1990). Not all of the LCMV strains necessarily ablate autoimmune diabetes in NOD mice. For instance, certain strains of LCMV, such as Armstrong 53b, Taub We, and Pasteur, can prevent diabetes, while an Armstrong 53b variant and Clone 13 fail to abort the disease. Analysis using genetic reassortants between the therapeutic strain of LCMV Pasteur and the nontherapeutic strain of LCMV Clone 13 has demonstrated that prevention of diabetes maps to the small (S) RNA segment of LCMV Pasteur (Oldstone *et al.*, 1990). The mechanism by which the S RNA segment of LCMV acts on infected CD4 T lymphocytes and then prevents the disease is not known. However, these observations suggest a potential use of viruses as therapeutic agents in treating IDDM.

E. Expression of Appropriate Class II MHC Molecules

The breeding study between NOD and I-E-transgenic B6 mice has shown that I-E expression can prevent autoimmune insulitis and diabetes in NOD mice (Nishimoto *et al.*, 1987). Subsequently, a similar backcross study (Böhme *et al.*, 1990) and production of transgenic NOD mice expressing I-E, I-A^k, or mutated $A\beta^{g7}$ (Uehira *et al.*, 1989; Miyazaki *et al.*, 1990; Slattery *et al.*, 1990; Lund *et al.*, 1990b) have demonstrated that prevention or reduction of insulitis and diabetes can be mediated by expression of appropriate I-A and I-E molecules.

These phenomena will be a clue in elucidating the mechanism of autoimmunity in NOD mice. Although the precise mechanism of disease prevention in class II transgenics is not known, there are several possibilities. First, in the case of transgenics expressing the mutated $A\beta^{g7}$ (Lund *et al.*, 1990b), the products of the transgenes compete with endogenous $A\beta^{g7}$ for $A\alpha^{g7}$, which may interfere with the presentation of β cell antigens to autoreactive T lymphocytes. However, I-E or I-A^k expression in transgenic NOD mice does not affect the expression of endogenous I-A^{g7}.

Second, I-E, or I-A^k molecules may compete with I-A^{g7} for binding to diabetogenic epitopes of islet cell antigens. Sette *et al.* (1989) have analyzed a panel of synthetic peptides for their binding capacities to I-A^d, I-A^k, I-E^d, and I-E^k. Their results suggest that different alleles of the same class II isotype may actually recognize closely related structures, whereas different isotypes may recognize albeit nonmutually exclusive structures on an antigenic molecule. However, prevention of insulitis and diabetes is more complete in I-E transgenics than in I-A^k transgenics (Nishimoto *et al.*, 1987; Uehira *et al.*, 1989; Miyazaki *et al.*, 1990; Lund *et al.*, 1990b; Slattery *et al.*, 1990). Moreover, spleen cells from (CBA × NOD) F_1 and I-E-transgenic NOD mice can present islet cell antigens to islet-reactive T cells (Haskins *et al.*, 1989; Nakano *et al.*, 1991). Therefore, a competition between I-A^{g7} and exogenous class II molecules for diabetogenic epitopes may not be sufficient to explain the I-E and I-A^k-mediated protection against diabetes.

Third, diabetogenic autoreactive T lymphocytes may be deleted or inactivated by I-E, I-A^k, or mutated I-A^{g7} expressed by transgenics. T lymphocytes expressing several V_β gene segments, including $V_\beta 5$, $V_\beta 11$, $V_\beta 12$, $V_\beta 16$, and $V_\beta 17a$, are known to be deleted in mice expressing I-E molecules (Kappler *et al.*, 1987; Bill *et al.*, 1988, 1989; Vacchio and Hodes, 1989). $V_\beta 5$- and $V_\beta 16$-positive cells, but not $V_\beta 11$- and $V_\beta 12$-positive cells, are deleted in I-E-expressing NOD mice (Lund *et al.*, 1990b; Böhme *et al.*, 1990; Nakano *et al.*, 1991; H. Kikutani and N. Nakano, unpublished). Diabetogenic T lymphocytes may utilize $V_\beta 5$ or $V_\beta 16$ gene segments or cross-react with I-E molecules in their fine specificities. In fact, one of the islet-reactive and disease-inducible T lymphocyte clones utilizes the $V_\beta 16$ gene segments (Nakano *et al.*, 1991). It is also noteworthy that induction of allogeneic tolerance by injecting F_1 semiallogeneic spleen cells into neonatal NOD mice results in a significant protection against both insulitis and diabetes (Bendelac *et al.*, 1989). Recently, it was reported that transfer of NOD bone marrow to irradiated (NOD × NON) F_1 mice can induce diabetes, whereas a mixture of NOD and NON or NOD.NON $H\text{-}2^{nb1}$ fails to transfer the disease (Serreze and Leiter, 1991). This also suggests allogeneic tolerance or disturbances of the emerging T cell repertoire. However, Böhme *et al.* (1990) have shown that I-E-mediated protection from diabetes is dissociated from clonal deletion of T lymphocytes. They have backcrossed transgenic mice carrying the wild-type or the promoter-mutated $E\alpha$ gene with NOD mice and have observed prevention of the disease in those mice carrying the wild-type $E\alpha$ genes, which express I-E molecules normally, but not in mice carrying the promoter-mutated $E\alpha$ genes, which express I-E molecules

only in particular compartments of the immune system. In contrast, a substantial decrease of $V_\beta 5$-expressing T lymphocytes has been observed in mice carrying the promoter-mutated $E\alpha$ genes as well as the wild-type genes. This result suggests that I-E-mediated protection against diabetes may involve more complex mechanisms. One of the major peptides bound to I-A molecules on B lymphocytes expressing I-A and I-E has been shown to be derived from I-E molecules (Rudensky *et al.*, 1991). Thus, there is also a possibility that such peptides presented by I-A expressed on antigen-presenting cells in the thymus or the periphery may influence the emergence of diabetogenic T lymphocytes in I-E-transgenic NOD mice.

Fourth, involvement of molecular mimicry may be able to explain prevention of diabetes in class II-transgenic NOD mice. It has been suggested that certain microbial agents that share antigenic epitopes with self antigens may trigger autoimmunity (Fujinami and Oldstone, 1985). It is also reasonable to assume the possibilty that a putative nonislet antigen may share a diabetogenic epitope with an islet antigen and may initiate autoimmunity by mimicking such an autoantigen in NOD mice as well. If other nondiabetogenic epitopes of this nonislet antigen presented by I-E or I-A^k or mutated I-A^{g7} are more immunogenic than a diabetogenic epitope presented by I-A^{g7}, the dominant T lymphocyte response may lessen the response to a diabetogenic epitope in class II-transgenic NOD mice. At present, we do not know which (if any) of these possibilities is the case. The identification of an islet antigen that really initiates autoimmunity will be necessary to explain this phenomenon.

VII. Concluding Remarks

The NOD mouse is an ideal model of organ-specific autoimmune diseases as well as IDDM. As described above, a large number of studies have been done on NOD mice, and some of them have contributed greatly to the understanding of both genetic and immunological control of IDDM.

One of the disease susceptibility genes is known to be linked to the MHC class II region in IDDM of humans, rats, and mice. Breeding and transgenic studies have demonstrated that both I-A^{g7} and an absence of I-E are necessary for development of autoimmune insulitis and diabetes. This indicates a difference as well as a similarity between mouse and human IDDM, since nonAsp 57 of the DQβ chain, but not a defect of HLA-DR molecules, equivalent to mouse I-E is observed in most cases of human IDDM. Further production and analyses of transgenic NOD mice carrying mutated genes should pinpoint a diabetogenic structure of the class II I-A molecule and such information will help to elucidate the role of particular class II molecules in the emergence of autoreactive T lymphocytes.

Several non-MHC-linked susceptibility genes have been recently mapped. These genes are suggested to play roles at different steps of the progression from initiation of autoimmune insulitis to islet destruction and overt diabetes. Mapping of these genes in NOD mice should also contribute to the identification of the human counterparts, because of the similarity between mouse and human chromosomes. Advances in gene and chromosome walking will make it possible to clone these genes in the near future. The characterization of such diabetogenic defects will accelerate studies on the etiopathogenesis of IDDM.

The development of IDDM in NOD mice can be divided roughly into two phases: initiation of autoimmune insulitis and promotion of islet destruction and overt diabetes. The former may represent a typical organ-specific autoimmune status and the latter may involve a dysregulation of systemic immune functions. It can be envisaged that information obtained from experimentally induced organ-specific autoimmune diseases such as EAE and collagen-induced arthritis could be adopted to studies of autoimmune insulitis of NOD. However, analyses of islet-infiltrating and islet-reactive T lymphocytes have revealed that autoimmune insulitis in NOD mice is much more complex than that in experimentally induced autoimmune diseases. Particularly, TCR usage of islet-reactive T lymphocyte clones has been found to be heterogeneous. These finding have raised two possibilities: (1) multiple autoantigens and polyclonal autoreactive T lymphocytes may be involved in the initiation of autoimmune insulitis, or (2) certain epitopes of autoantigens and respective T cells trigger autoimmune insulitis and quickly recruit secondary, polyclonal autoreactive T lymphocytes reactive to other self determinants. We do not know what autoantigens are recognized by islet-reactive or diabetogenic T lymphocytes. Recently, GAD has been identified as one of the target antigens of islet cell autoantibodies in human, rat, and mouse IDDM. It will be interesting to know if GAD is also recognized by autoreactive T lymphocytes in NOD mice. Identification of antigenic epitopes recognized by islet-reactive T lymphocytes and fine mapping of the functional structure of I-A^{g7} required for presenting such T cell epitopes may provide clues for intervention in the development of IDDM as well as for understanding the mechanism of autoimmunity in NOD mice.

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The Pathobiology of Bronchial Asthma

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I. Introduction

Bronchial asthma is a disease that is characterized by a history of episodic wheezing, by physiologic evidence of reversible airflow obstruction, either spontaneously or following bronchodilator therapy, and by pathologic evidence of inflammatory changes in the bronchial mucosa. Asthma is a common disease and there is accumulating evidence that it may be growing in prevalence, thereby imposing an increasingly large burden on the health services and claiming ever greater numbers of lives each year.

Against the epidemiologic background, it is clear that elucidation of the mechanisms for the development and perpetuation of the asthmatic diathesis is critical. In recent years there has been a major change in the conceptual basis of the pathophysiology of this disease. This has resulted from the recognition that inflammation of the airways is a characteristic feature of asthma and that obstruction of the airway lumen by smooth muscle constriction and mucus plugging may be the sequelae of the inflammatory cascades. This view has led to a significant change in the focus of research activities and in ideas about therapy of asthma. The present review will include a summary of the recent developments in research on the pathobiology of the disease. The focus will be on the cellular mechanisms of the disease as indicated by current information on the pathologic changes in the asthmatic bronchial mucosa obtained in living patients via the fiberoptic bronchoscope. Also addressed are the mediator mechanisms, with special consideration of the possible role of the sulfidopeptide leukotrienes, prostanoid metabolites, and platelet-activating factor. Bronchial asthma is not a homogeneous disease and there are well-defined subgroups of patients. Heterogeneity in asthmatic syndromes will be addressed by a consideration of two well-defined clinical subgroups, namely, aspirin-induced asthma and corticosteroid-resistant bronchial asthma.

Exhibit I

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The Non Obese Diabetic (NOD) mouse, as a model of T cell mediated autoimmune disease

Jean-François BACH

La souris NOD (« Non Obese Diabetic »), modèle de maladie autoimmune à médiation cellulaire

Résumé — Les souris « Non Obese Diabetic » (NOD) représentent un précieux modèle de diabète insulinodépendant humain. Les études réalisées chez ces souris ont montré que le diabète est lié à l'action exclusive des cellules T (CD4) et CD8). La maladie est déterminée par la présence simultanée de plusieurs gènes. Deux d'entre eux ont été localisés : un gène H-2 du complexe majeur d'histocompatibilité, sur le chromosome 17 et un gène codant pour la périinsulite sur le chromosome 1. La maladie est favorisée par un dérèglement des fonctions régulatrices, notamment de certains mécanismes suppresseurs faisant intervenir les cellules CD4. La souris NOD est utilisée avec succès pour rechercher de nouveaux traitements immunopréventifs applicables à l'homme.

Type I diabetes is an autoimmune disease characterized by the presence of anti-islet cell antibodies, association to other autoimmune diseases and defined HLA antigens and sensitivity to immunosuppressive therapy. Two animal models of the disease have been reported: the BB rat and the NOD mouse.

The NOD mouse shows infiltration of islets by mononuclear cells at 4-6 weeks of age and clinical diabetes at 4-6 months with a clear predominance in females. This sex difference in disease incidence and severity is due to the protective effect of androgens as shown by castration experiments. It is a T cell mediated disease as assessed by disease transfer achieved by T cell transfusion. Both $CD4^+$ and $CD8^+$ T cells are required to obtain the transfer which can be achieved inasmuch as the recipient is immunocompetent (neonate or irradiated adult) [1]. Injection of purified CD4 and CD8 populations shows that $CD4^+$ cells enter the pancreas in which they can penetrate in absence of $CD8^+$ cells. Conversely $CD8^+$ cells require the presence of $CD4^+$ cells to enter the pancreas [2]. Some data argue in favor of a restriction of V β gene usage by diabetogenic T cells, notably data obtained in our laboratory by Claude Carnaud for the V β6 gene, but the point remains controversial. The nature of the effector mechanism which leads to β cell destruction is also to be determined: lymphokine production or cytolysis mechanism? The answer will probably have to await the identification of the target autoantigens which is not yet defined in spite of the proposal of several candidate autoantigens (a 64K protein, shown to be glutamic acid decarboxylase [3], a 58K [4] and a 38K [5] proteins, gangliosides, etc.).

The genetic control of diabetes in NOD mice has been the matter of extensive investigation. The role of MHC genes is well established. Specific I-A encoded antigens are mandatory for islet cell autoantigen recognition. The absence of I-E gene observed in NOD mice could also play a role since I-E transgenic mice are protected from diabetes

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onset [6]. Segregation studies of F2 crosses and backcrosses indicate however that non MHC genes also intervene. Studies by Wicker [7] and Leiter [8] have indeed shown the existence of such genes. Recently, in collaboration with H. J. Garchon we have identified in (NOD × C57BL/6) F2 mice one of these genes which codes for peri insulitis, the first phase of the disease [9]. Using microsatellite probes we have mapped this gene to chromosome 1 close to the Bcl2 oncogene. Its mechanism of expression is not known (influence on lymphocyte homing?) More generally much remains to be learnt on the mechanisms of expression of diabetes predisposing genes.

It is likely that one or several of these genes code for the immune disregulation observed in these mice. One may thus assume that the defective suppressor cell control is under genetic control. Such a defect is indicated by the acceleration of the disease afforded by thymectomy and cyclophosphamide, two procedures known to alter suppressor cell function. Direct evidence for the existence of diabetes suppressor CD4 T cells has been obtained in a transfer model [10]. We do not know however the precise phenotype of the suppressor cells nor its T cell receptor gene usage or its mode of action (lymphokine production?).

All these observations have important clinical therapeutic applications. In fact, one may prevent the onset of diabetes in NOD mice by a number of methods including chemicals (cyclosporine, FK 506) or monoclonal antibodies (anti T cell receptor, anti-CD3, CD4, IL-2 receptor antibodies). Several of these methods have already been applied to human insulin dependent diabetes and one may hope that the strategies established in NOD mice will permit us to set up protocols leading ultimately to immunoprevention of the disease in man.

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Exhibit J

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Activators and target genes of Rel/NF-κB transcription factors

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The vertebrate transcription factor NF-κB is induced by over 150 different stimuli. Active NF-κB, in turn, participates in the control of transcription of over 150 target genes. Because a large variety of bacteria and viruses activate NF-κB and because the transcription factor regulates the expression of inflammatory cytokines, chemokines, immunoreceptors, and cell adhesion molecules, NF-κB has often been termed a 'central mediator of the human immune response'. This article contains a complete listing of all NF-κB inducers and target genes described to date. The collected data argue that NF-κB functions more generally as a central regulator of stress responses. In addition, NF-κB activation blocks apoptosis in several cell types. Coupling stress responsiveness and anti-apoptotic pathways through the use of a common transcription factor may result in increased cell survival following stress insults.

Keywords: NF-κB; Rel; transcription factors; immune response; stress response; ER overload

NF-κB, a central mediator of the human immune response

The Rel/NF-κB family of eukaryotic transcription factors is comprised of several structurally-related proteins that form homodimers and heterodimers (Chen and Ghosh, 1999, this issue). In vertebrates, this family includes p50/p105, p52/p100, RelA (p65), c-Rel and RelB. These dimers bind to a set of related 10 bp DNA sites, collectively called κB sites, to regulate the expression of many genes. In most cells, Rel/NF-κB transcription complexes are present in a latent, inactive state in the cytoplasm where they are bound to an inhibitor (IκB). As described below, many stimuli can rapidly activate these transcription complexes by freeing them from their inhibitor and enabling them to translocate to the nuclues. The most common Rel/NF-κB dimer in mammals contains p50-RelA and is specifically called NF-κB. For the purposes of this review, NF-κB will be used to refer any induced complex that can be translocated from the cytoplasm to the nucleus and can bind to κB sites.

The transcription factor NF-κB has often been called a 'central mediator of the human immune response'. How was such a reputation established and is it justified? A summary of all stimuli that are known to activate NF-κB (Table 1) and a compilation of its many target genes (Table 2) may provide an answer.

In many cell types, nuclear NF-κB activity is induced by exposure to a wide variety of bacteria or bacterial products (Table 1). Likewise, a host of viruses or their proteins activate NF-κB (Table 1). Bacterial and viral infection certainly present situations where an adequate immune response is vital. That human cells respond to so many different organisms by activating the same transcription factor, NF-κB, is one reason for its reputation as a 'central switch'. Moreover, homozygous disruption in mice of the genes encoding certain members of the Rel/NF-κB family, including those encoding c-Rel, p50 and RelB, leads to defects in the immune response to certain pathogens (Gerondakis *et al.*, 1999, this issue).

The active NF-κB transcription factor promotes the expression of over 150 target genes (Table 2). The majority of proteins encoded by NF-κB target genes participate in the host immune response. These include, for example, 27 different cytokines and chemokines, as well as receptors required for immune recognition, such as MHC molecules, proteins involved in antigen presentation and receptors required for neutrophil adhesion and transmigration across blood vessel walls (Table 2). These target genes alone would merit NF-κB the designation as a 'central mediator of the immune response'.

Many viruses that induce NF-κB activity also harbor NF-κB binding sites in their viral promoters (Table 2). Therefore, it seems likely that a virus would gain a selective advantage from the acquisition of a κB site in its promoter. If the transcription factor is induced either directly through viral infection or indirectly by the ensuing immune response (via inflammatory cytokines, for example), the κB site-containing viral promoter will be transactivated, resulting in enhanced viral transcription. Thus, the organism's own sword is turned against itself. The presence of a κB site in the HIV-1 promoter may have led to the activation of viral replication that was observed during trials in which IL-2 was used to stimulate T-cell replication in HIV-1-infected patients (Kovacs *et al.*, 1995). A low level of NF-κB activation is perhaps part of the mechanism by which some viruses, such as EBV, HSV, CMV or HIV-1, maintain their chronic infections.

A compilation of the many pathogens that induce NF-κB and a look at the function of its various target genes certainly validate the reputation this transcription factor has gained as an important regulator of the immune response. Moreover, the fact that viruses often use this protein to their advantage argues that NF-κB activity exerted an evolutionary pressure on these pathogens.

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Table 1 Inducers of NF-κB activity

Condition	*Reference*
Bacteria	
EPEC, enterophathogenic *E. coli*	Savkovic *et al.*, 1997
Gardnerella vaginalis	Hashemi *et al.*, 1999
Helicobacter pylori	Münzenmaier *et al.*, 1997
Lactobacilli	Klebanoff *et al.*, 1999
Listeria monocytogenes	Hauf *et al.*, 1994
Micoplasma fermentans	Marie *et al.*, 1999
Mycobacteria tuberculosis	Zhang *et al.*, 1994
Neisseria gonorrhoeae	Naumann *et al.*, 1997
Rickettsia rickettsii	Sporn *et al.*, 1997
Salmonella dublin	Eaves-Pyles *et al.*, 1999
Salmonella typhimurium	Hobbie *et al.*, 1997
Shigella flexneri	Dyer *et al.*, 199
Staphyllococcus aureus	Busam *et al.*, 1992
Bacterial Products	
Diphosphoryl lipid A (*Rhodobacter sphaeroides*)	Lawrence *et al.*, 1995
Exotoxin B	Busam *et al.*, 1992
G(Anh) M Tetra	Dokter *et al.*, 1994
Lipoteichoic acid (*Listeria*)	Hauf *et al.*, 1997
Lipopolysaccharide (LPS)	Sen and Baltimore, 1986a
membrane lipoproteins (*Micoplasma fermentans*)	Garcia *et al.*, 1998; Rawadi *et al.*, 1999
Muramyl Peptides	Schreck *et al.*, 1992
PlcA (Phospholipase) (*Listeria*)	Hauf *et al.*, 1997
PlcB (Phospholipase) (*Listeria*)	Hauf *et al.*, 1997
Staphylococcus enterotoxin A and B (super antigen)	Trede *et al.*, 1993; Busam *et al.*, 1992
Toxic Shock Syndrome Toxin 1	Trede *et al.*, 1993
Viruses	
Adenovirus	Shurman *et al.*, 1989
Cytomegalovirus	Sambucetti *et al.*, 1989
Epstein-Barr Virus (EBV)	Hammarskjöld and Simurda, 1992
Hepatitis B Virus	Siddiqui *et al.*, 1989
Herpes Virus Saimiri	Yao *et al.*, 1995
Human Herpesvirus 6	Ensoli *et al.*, 1989
HIV-1	Bachelerie *et al.*, 1991
Herpex Simplex Virus -1	Gimble *et al.*, 1988
HTLV-I	Leung and Nabel, 1988; Ballard *et al.*, 1988
Influenza Virus	Ronni *et al.*, 1997
Measles Virus	Harcourt *et al.*, 1999
Molony Murine Leukemia Virus	Pak and Faller, 1996
Newcastle disease virus	Ten *et al.*, 1993
Respiratory Syncytial Virus	Mastronarde *et al.*, 1996; Garofalo *et al.*, 1996
Rhinovirus	Zhu *et al.*, 1996a; Zhu *et al.*, 1996b
Sendai paramyxo virus	Hiscott *et al.*, 1989
Sindbis Virus	Lin *et al.*, 1995a
Viral Products	
Adenovirus 5: E1A	Shurman *et al.*, 1989
Adenovirus: E3/19K	Pahl *et al.*, 1996
CMV: ie1	Sambucetti *et al.*, 1989
Double-stranded RNA	Visvanathan and Goodbourn, 1989
EBV: EBNA-2	Scala *et al.*, 1993
EBV: LMP	Hammarskjöld and Simurda, 1992
HBV: HBx	Twu *et al.*, 1989
HBV: LHBs	Hildt *et al.*, 1996
HBV: MHBs^t	Meyer *et al.*, 1992
HCV: Core protein	You *et al.*, 1999
Herpes Saimiri: HVS13	Yao *et al.*, 1995
HIV-1: gp160	Chirmule *et al.*, 1994
HIV-1: Tat	Westendorp *et al.*, 1994
HTLV-I: Tax1	Ballard *et al.*, 1988; Leung and Nabel, 1988
HTLV-II: Tax2	Tanaka *et al.*, 1996
Influenza Virus: Hemagglutinin	Pahl and Baeuerle, 1995a
Parvovirus B19: NSI	Moffatt *et al.*, 1996
Eukaryotic parasite	
Theileria parva	Ivanov *et al.*, 1989
(Inflammatory) Cytokines	
IL-1	Osborn *et al.*, 1989
IL-2	Hazan *et al.*, 1990
IL-12	Grohmann *et al.*, 1998
IL-15	McDonald *et al.*, 1998
IL-17	Shalom-Barak *et al.*, 1998
IL-18	Matsumoto *et al.*, 1997
LIF	Gruss *et al.*, 1992
THANK	Mukhopadhyay *et al.*, 1999
TNFα	Osborn *et al.*, 1989; Israël et al., 1989a
TNFβ	Messer *et al.*, 1990
Physiological (Stress) Conditions	
Adhesion	Lin *et al.*, 1995b
Depolarization	Kaltschmidt *et al.*, 1995
Hemorrhage	Shenkar *et al.*, 1996; Shenkar and Abraham, 1997
Hyperglycemia	Yerneni *et al.*, 1999
Hyperosmotic Shock	Courtois *et al.*, 1997
Hyperoxia	Shea *et al.*, 1996
Ischemia (transient, focal)	Gabriel *et al.*, 1999; Li *et al.*, 1999
Liver Regeneration	Tewari *et al.*, 1992; Cressman *et al.*, 1994
Mechanical Ventilation (*in vitro*)	Pugin *et al.*, 1998
Reoxygenation	Rupec and Baeuerle, 1995
Shear Stress	Lan *et al.*, 1994
T-cell Selection	Moore *et al.*, 1995
Physical Stress	
PPME Photosensitization	Legrand-Poels *et al.*, 1995
Ultraviolet irradiation (UV-A, B, C)	Stein *et al.*, 1989
Wounding combined with HeNe irradiation	Haas *et al.*, 1998
γ Radiation	Brach *et al.*, 1991a
Oxidative Stress	
Butyl Peroxide	Munroe *et al.*, 1995
Hydrogen Peroxide	Schreck *et al.*, 1991
Ozone	Haddad *et al.*, 1996
Pervanadate	Imbert *et al.*, 1996
Reoxygenation	Rupec and Baeuerle, 1995
Environmental Hazards	
3,3',4,4'-tetrachlorobiphenyl (PCB77)	Hennig *et al.*, 1999
Chromium	Ye *et al.*, 1995
Cigarette Smoke	Nishikawa *et al.*, 1999
Cobalt	Goebeler *et al.*, 1995
Crocidolite asbesstos fibres	Janssen et al., 1995
Dicamba (herbicide, peroxisome proliferator)	Espandiari *et al.*, 1998
Lead	Ramesh *et al.*, 1999
Nickel	Goebeler *et al.*, 1995
Silica Particles	Chen *et al.*, 1995
Therapeutically used drugs	
1-b-D-Arabinofuranosyl-cytosine (ara-C)	Strum *et al.*, 1994
Anthralin	Schmidt *et al.*, 1996
Azidothymidine (AZT)	Kurata, 1994
Camptothecin	Piret and Piette, 1996
Ciprofibrate	Li *et al.*, 1996a
Cisplatin	Nie *et al.*, 1998
Daunomycin	Das and White, 1997; Hellin *et al.*, 1998
Daunorubicin	Wang *et al.*, 1996
Doxorubicin	Das and White, 1997
Etoposide	Bessho *et al.*, 1994
Haloperidol	Post *et al.*, 1998
Methamphetamine	Asanuma and Cadet, 1998
Phenobarbital	Li *et al.*, 1996b
Tamoxifen	Ferlini *et al.*, 1999

continued

Table 1 continued

Condition	Reference
Taxol (Paclitaxel)	Hwang and Ding, 1995
Vinblastine	Rosette and Karin, 1995a
Vincristine	Das and White, 1997
Modified Proteins	
Advanced glycated end products (AGEs)	Yan *et al.*, 1994; Wautier *et al.*, 1994
Amyloid Protein Fragment (βA4)	Behl *et al.*, 1994
Maleylated BSA	Misra *et al.*, 1996
Modified (Oxidized)LDL	Rajavashisth *et al.*, 1995; Andalibi *et al., 1993*
Overexpressed Proteins (ER Overload)	
CFTR	Knorre and Pahl, unpublished observation
Erythropoietin-Receptor	Knorre and Pahl, unpublished observation
Ig heavy chain	Pahl and Baeuerle, 1995b
MHC Class I	Pahl and Baeuerle, 1995b
Receptor Ligands	
Antigen (IgM-Ligand)	Marcuzzi *et al.*, 1989
CD11b/CD18-Ligand (Complement)	Thieblemont *et al.*, 1995
CD28-Ligand (B7-1)	Verweij *et al.*, 1991
CD2-Ligand	Bressler *et al.*, 1991
CD35-Ligand (Complement)	Thieblemont *et al.*, 1995
CD3-Ligand	Tong-Starksen *et al.*, 1989
CD40-Ligand	Berberich *et al.*, 1994
CD4-Ligand (gp120)	Chirmule *et al.*, 1994
Fc-2a-Receptor-Ligand (IgG2a)	Muroi *et al.*, 1994
Flt-1-Ligand	Reikerstorfer *et al.*, 1995
Ly6A/E-Ligand	Ivanov *et al.*, 1994
N-CAM	Krushel *et al.*, 1999
Trail-receptor-1-Ligand (Trail)	Schneider *et al.*, 1997
Trail-receptor-2-Ligand (Trail)	Schneider *et al.*, 1997
Trail-receptor-4-Ligand (Trail)	Degli-Esposti *et al.*, 1997
Apoptotic Mediators	
Anti-Fas/Apo-1	Rensing-Ehl *et al.*, 1995
Trail	Schneider *et al.*, 1997
Mitogens, growth factors and hormones	
Bone morphogenic protein 2	Mohan *et al.*, 1998
Bone morphogenic protein 4	Mohan *et al.*, 1998
Folicle Stimulating Hormone	Delfino and Walker, 1998
Human Growth Hormone	Shen *et al.*, 1997
Insulin	Bertrand *et al.*, 1995
M-CSF	Brach *et al.*, 1991b
Nerve Growth Factor	Wood, 1995; Carter *et al.*, 1996
Platelet-Derived Growth Factor	Olashaw *et al.*, 1992
Serum	Baldwin *et al.*, 1991
TGF-α	Lee *et al.*, 1995
Physiological Mediators	
12(R)-Hydroxyeicosatrienoic acid	Laniado-Schwartzman *et al.*, 1994
Amino acid analogs	Kretz-Remy *et al.*, 1998
Anaphylatoxin C3a	Pan, 1998
Anaphylatoxin C5a	Pan, 1998
Angiotensin II	Li and Brasier, 1996
Basic calcium phosphate crystals	McCarthy *et al.*, 1998
Bradikinin	Pan *et al.*, 1996
C2-Ceramide (N-acetyl-sphingosine)	Andrieu *et al.*, 1995
Cerulein	Gukovsky *et al.*, 1998; Steinle *et al.*, 1999
Collagen lattice	Xu *et al.*, 1998
Collagen Type I	Lee *et al.*, 1995
Des-Arg10-kallidin (B1 receptor agonist)	Schanstra *et al.*, 1998
Double-stranded polynucleotides	Suzuki *et al.*, 1999
f-Met-Leu-Phe	Browning *et al.*, 1997
Heat shock protein 60 (HSP 60)	Kol *et al.*, 1999
Hemoglobin	Simoni *et al.*, 1998
Hyaluronan	Noble *et al.*, 1996
Kaianic acid (Kainate)	Kaltschmidt *et al.*, 1995
Leukotriene B4	Brach *et al.*, 1992
L-Glutamate	Guerrini *et al.*, 1995
Lysophosphatidylcholine (LysoPC)	Zhu *et al.*, 1997
PAF (platelet activating factor)	Smith and Shearer, 1994; Mutoh *et al.*, 1994
Potassium	Kaltschmidt *et al.*, 1995
Thrombin	Mari *et al.*, 1994
Chemical Agents	
2-Deoxyglucose	Pahl and Baeuerle, 1995b
Anisomycin	Sen and Baltimore, 1986a
Brefeldin A	Pahl and Baeuerle, 1995b
Calchicine	Rosette and Karin, 1995a
Calcium Ionophores	Novak *et al.*, 1990
Calyculin A	Suzuki *et al.*, 1994
Cobalt chloride	Sultana *et al.*, 1999
Con A	Rattner *et al.*, 1991
Cycloheximide	Sen and Baltimore, 1986a
Cyclopiazonic Acid	Pahl *et al.*, 1996
Forskolin	Delfino and Walker, 1998
Glass fibres	Ye *et al.*, 1999
Linoleic acid	Hennig *et al.*, 1996
L-NMA	Peng *et al.*, 1995
Lysophosphatidic acid	Shahrestanifar *et al.*, 1999
Monensin	Pahl and Baeuerle unpublished observation
N-methyl-D-aspartate	Guerrini *et al.*, 1995
Nocodazol	Rosette and Karin, 1995a
Okadaic Acid	Thevenin *et al.*, 1991
PHA	Sen and Baltimore, 1986b
Phorbol ester	Sen and Baltimore, 1986a
Podophyllotoxin	Rosette and Karin, 1995a
Pyrogallol	Adcock *et al.*, 1994
Quinolinic acid	Qin *et al.*, 1998
Thapsigargin	Pahl *et al.*, 1996
Tunicamycin	Pahl and Baeuerle, 1995b
Vinblastine	Rosette and Karin, 1995a

Where possible, the first publication to report the data is given as a reference

NF-κB, a central regulator of the stress response

NF-κB, however, is involved in the control of transcription of many genes whose functions extend beyond the immediate immune response. For example, NF-κB also regulates the transcription of many acute phase proteins (Table 2). Similarly, there are many activators of NF-κB that are not bacterial and viral pathogens. Therefore, rather than being a central mediator of the immune response, NF-κB perhaps more generally represents a regulator of stress responses. NF-κB activity, for instance, is induced during various physiological stress conditions such as ischemia/reperfusion, liver regeneration and hemorrhagic shock (Table 1). Physical stress in the form of irradiation as well as oxidative stress to cells also induce NF-κB (Table 1). In this context, it appears evolutionarily beneficial that a large variety of stress response genes, such as the inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), are in turn activated by NF-κB (Table 2). Again, NF-κB relays the information of an imminent stress and at the same time enacts a response by promoting the transcription of genes whose products alleviate the stress condition.

Besides physiological stress situations, the human body is exposed to environmental hazards and therapeutic drugs, which can also exert a stress. Indeed, NF-κB is activated both by environmental stresses, such as heavy metals or cigarette smoke, and

Table 2 Target genes of NF-κB

Gene	*Function*	*Reference*
Cytokines/Chemokines and their modulators		
CINC	Cytokine-induced neutrophil chemoattractant	Blackwell *et al.*, 1994; Ohtsuka *et al.*, 1996
*CXCL 11	Chemokine ligand for CXCR3	Tensen *et al.*, 1999
Eotaxin	β Chemokine, eosinphil-specific	Hein *et al.*, 1997
Gro α-γ	Melanoma growth stimulating activity	Anisowicz *et al.*, 1991
IFN-γ	Interferon	Sica *et al.*, 1992; Sica *et al.*, 1997
IL-1α	Interleukin-1α	Mori and Prager, 1996
IL-1β	Interleukin-1β	Hiscott *et al.*, 1993
IL-1-receptor antagonist	Inhibitor of IL-1 activity	Smith *et al.*, 1994
IL-2	Interleukin-2	Serfling *et al.*, 1989; Hoyos *et al.*, 1989; Lai *et al.*, 1995
IL-6	Interleukin-6, inflammatory cytokine	Libermann and Baltimore, 1990; Shimizu *et al.*, 1990
IL-8	Interleukin-8, α-chemokine	Kunsch and Rosen, 1993
*IL-9	Interleukin-9	Zhu *et al.*, 1996a
IL-11	Interleukin-11	Bitko *et al.*, 1997
IL-12 (p40)	Interleukin-12	Murphy *et al.*, 1995
*IL-15	Interleukin-15	Azimi *et al.*, 1998
β-Interferon	Interferon	Hiscott *et al.*, 1989; Lenardo *et al.*, 1989
IP-10	α Chemokine	Ohmori and Hamilton, 1993
KC	α Chemokine	Ohmori *et al.*, 1995
Lymphotoxin α		Worm *et al.*, 1998
Lymphotoxin β	Anchors TNF to cell surface	Kuprash *et al.*, 1996
MCP-1/JE	Macrophage chemotactic protein, β Chemokine	Ueda *et al.*, 1994
MIP-1α,β	Macrophage inflammatory protein-1, β Chemokine	Grove and Plumbi, 1993; Widmer *et al.*, 1993
MIP-2	Macrophage inflammatory protein-1, β Chemokine	Widmer *et al.*, 1993
RANTES	Regulated upon Activation Normal T lymphocyte Expressed and Secreted, β Chemokine	Moriuchi *et al.*, 1997
TCA3, T-cell activation gene 3	T-cell activation gene 3, β Chemokine	Oh and Metcalfe, 1994
TNFα	Tumor necrosis factor α	Shakhov *et al.*, 1990; Collart *et al.*, 1990
TNFβ	Tumor necrosis factor β	Paul *et al.*, 1990; Messer *et al.*, 1990
Immunoreceptors		
B7.1 (CD80)	Co-stimulation of T cells via CD28 binding	Fong *et al.*, 1996; Zhao *et al.*, 1996
BRL-1	B-cell homing receptor	Wolf *et al.*, 1998
CCR5	Chemokine receptor	Liu *et al.*, 1998
CD48	Antigen of stimulated lymphocytes	Klaman and Thorley-Lawson, 1995
F_c epsilon receptor II (CD23)	Receptor for IgE	Richards and Katz, 1997
IL-2 receptor α-chain	IL-2 receptor subunit	Ballard *et al.*, 1988
Immunoglobulin Cgamma1	IgG heavy chaini	Lin and Stavnezer, 1996
Immunoglobulin ε heavy chain	IgE heavy chain	Iciek *et al.*, 1997
Immunoglobulin κ light chain	Antibody light chain	Sen and Baltimore, 1986b
Invariant Chain I_i	Antigen presentation	Pessara and Koch, 1990
MHC class I (H-2K^b)	Mouse histocompatibility antigen	Israël *et al.*, 1989a; Israël *et al.*, 1989b
MHC Class I HLA-B7	Mouse histocompatibility antigen	Johnson and Pober, 1994
β2 Microglobulin	Binds MHC class I	Israël *et al.*, 1989a; Israël *et al.*, 1989b
T-cell receptor β chain	T-cell receptor subunit	Jamieson *et al.*, 1989
*TNF-Receptor, p75/80	High-affinity TNF receptor	Santee and Owen-Schaub, 1996
Proteins involved in antigen presentation		
Proteasome Subunit LMP2	Subunit of 26S proteasome, cysteine protease	Wright *et al.*, 1995
Peptide Transporter TAP1	Peptide transporter for ER	Wright *et al.*, 1995
Cell adhesion molecules		
ELAM-1	E-selectin, endothelial cell leukocyte adhesion molecule	Whelan *et al.*, 1991
ICAM-1	Intracellular adhesion molecule-1	van de Stolpe *et al.*, 1994
MadCAM-1	Mucosal addressin cell adhesion molecule	Takeuchi and Baichwal, 1995
P-selectin	Platelet adhesion receptor	Pan and McEver, 1995
Tenascin-C	ECM protein controls cell attachment and migration, cell growth	Mettouchi *et al.*, 1997
VCAM-1	Vascular cell adhesion molecule	Iademarco *et al.*, 1992
Acute phase proteins		
Angiotensinogen	Angiotensin precursor, regulates blood pressure	Brasier *et al.*, 1990; Ron *et al.*, 1990
C4b binding protein	Complement binding protein	Moffat and Tack, 1992
Complement factor B	Complement factor	Nonaka and Huang, 1990
Complement Factor C4	Activates extrinsic pathway of complement activation	Yu *et al.*, 1989
C-reactive protein	Pentraxin	Zhang *et al.*, 1995
Lipopolysaccharide binding protein	Binds to LPS receptor (CD14) with LPS	Schumann, 1995
Pentraxin PTX3	Pentraxin	Basile *et al.*, 1997
Serum amyloid A precursor	Serum component	Edbrooke *et al.*, 1991; Li and Liao, 1991
Tissue factor-1	Activates extrinsic pathway of complement activation	Mackman *et al.*, 1991
Urokinase-type Plasminogen activator	Activates fibrinogen for fibrin clot lysis	Novak *et al.*, 1991
Stress response genes		
Angiotensin II	Peptide hormone	Brasier *et al.*, 1990

continued

Table 2 continued

Gene	Function	Reference
COX-2	Cyclooxygenase, prostaglandin endoperoxide synthase	Yamamoto *et al.*, 1995
Ferritin H chain	Iron storage protein	Kwak *et al.*, 1995
*5-Lipoxygenase	Arachidonic acid metabolic enzyme, leukotriene synthesis	Chopra *et al.*, 1992
12-Lipoxygenase	Arachidonic acid metabolic enzyme	Arakawa *et al.*, 1995
inducible NO-Synthase	NO synthesis	Geller *et al.*, 1993
Mn SOD	Superoxide dismutase	Das *et al.*, 1995
NAD(P)H quinone oxidoreductase (DT-diaphorase)	Bioreductive enzyme	Yao and O'Dwyer, 1995
Phospholipase A2	Fatty acid metabolism	Morri *et al.*, 1994
Cell-surface receptors		
A1 adenosine receptor	Pleiotropic physiological effects	Nie *et al.*, 1998
Bradikinin B1-Receptor	Pleiotropic physiological effects	Ni *et al.*, 1998
*CD23	Cell-surface molecule	Tinnell *et al.*, 1998
CD69	Lectin mainly on activated T cells	Lopez-Cabrera *et al.*, 1995
Gal1 Receptor	Galanine receptor, neuroendocrine peptide	Lorimer *et al.*, 1997
Lox-1	Receptor for Oxidized low density lipoprotein	Nagase *et al.*, 1998
Mdr1	Multiple drug resistance mediator (P-glycoprotein)	Zhou and Kuo, 1997
Neuropeptide Y Y1-receptor	Pleiotropic physiological effects	Musso *et al.*, 1997
PAF receptor 1	Platelet activator receptor	Mutoh *et al.*, 1994
RAGE- receptor for advanced glycation end products	Receptor for Advanced Glycation End products	Li and Schmidt, 1997
Regulators of apoptosis		
Bfl1/A1	Pro-survival Bcl-2 homologue	Grumont *et al.*, 1999; Zong *et al.*, 1999
Bcl-xL	Pro-survival Bcl-2 homologue	Chen *et al.*, 1999; Lee *et al.*, 1999b
Nr13	Pro-survival Bcl-2 homologue	Lee *et al.*, 1999
cCD95 (Fas)	Pro-apoptotic receptor	Chan *et al.*, 1999
Fas-Ligand	Inducer of apoptosis	Matsui *et al.*, 1998
IAPs	Inhibitors of Apoptosis	You *et al.*, 1997; Stehlik *et al.*, 1998
IEX-1L	Immediate early gene	Wu *et al.*, 1998
Growth factors and their modulators		
G-CSF	Granulocyte Colony Stimulating Factor	Nishizawa and Nagata, 1990
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor	Schreck and Baeuerle, 1990
*IGFBP-1	Insulin-like growth factor binding protein-1	Lang *et al.*, 1999
IGFBP-2	insulin-like growth factor binding protein-2	Cazals *et al.*, 1999
M-CSF (CSF-1)	Macrophage Colony Stimulating Factor	Brach *et al.*, 1991b
PDGF B chain	Platelet-Derived Growth Factor	Khachigian *et al.*, 1995
Proenkephalin	Hormone	Rattner *et al.*, 1991
*Thrombospondin	Matrix glycoprotein t	Adolph *et al.*, 1997
VEGF C	Vascular Endothelial Growth Factor	Chilov *et al.*, 1997
Early response genes		
p22/PRG1	Rat homology of IEX	Schafer *et al.*, 1998*
p62	Non-proteasomal multi-ubiquitin chain binding protein	Vadlamudi and Shin, 1998
Transcription factors		
A20	TNF-inducible zinc finger	Krikos *et al.*, 1992
c-*myb*	Proto-oncogene	Toth *et al.*, 1995
c-*myc*	Proto-oncogene	Duyao *et al.*, 1992
c-*rel*	Proto-oncogene	Hannink and Temin, 1990
IRF-1	Interferon regulatory factor-1	Harada *et al.*, 1994
IRF-2	Interferon regulatory factor-2	Harada *et al.*, 1994
IκBα	Inhibitor of Rel/NF-κB	Haskill *et al.*, 1991; Sun *et al.*, 1993; deMartin *et al.*, 1993
*jun*B	Proto-oncogene	Brown *et al.*, 1995
nfkb2	NF-κB p100 precursor	Lombardi *et al.*, 1995
nfkb1	NF-κB p105 precursor	Ten *et al.*, 1992
p53	Tumor suppressor	Wu and Lozano, 1994
Viruses		
Adenovirus (E3 region)	Adenovirus	Williams *et al., 1990*
Avian Leukosis Virus	Causes avian leukosis	Bowers *et al.*, 1996
Bovine Leukemia Virus	Causes bovine leukemia	Brooks *et al.*, 1995
CMV	Cytomegalovirus	Sambucetti *et al.*, 1989
EBV (Wp promoter)	Epstein-Barr virus	Sugano *et al.*, 1997
HIV-1	Human immunodeficiency virus	Nabel and Baltimore, 1987; Griffin *et al.*, 1989
HSV	Herpes simplex virus	Rong *et al.*, 1992
JC Virus	Polyoma virus	Ranganathan and Khalili, 1993
Measles virus	Causes measles	Harcourt *et al.*, 1999
SIV	Simian immunodeficiency virus	Bellas *et al.*, 1993
SV-40	Simian virus 40	Kanno *et al.*, 1989
Enzymes		
*Ceramide glycosyl transferase	Glycosphingolipid	Ichikawa *et al.*, 1998
Collagenase 1	Matrix metalloproteinase	Vincenti *et al.*, 1998

continued

Table 2 continued

Gene	*Function*	*Reference*
*Dihydrodiol dehydrogenase	Oxidoreductase, oxidation of trans-hydodiols	Ciaccio *et al.*, 1996
*GAD67	Glutamic acid decarboxylase	Szabo *et al.*, 1996
Gelatinase B	Matrix metalloproteinase	He, 1996
GSTP1-1	Glutathione transferase	Xia *et al.*, 1996*
Glucosel-6-phosphate dehydrogenase	Hexose monophosphate	Garcia-Nogales *et al.*, 1999
* HO-1	Hemeoxygenase	Lavrovsky *et al.*, 1994
Hyaluronan synthase	Synthesizes hyaluronic acid	Ohkawa *et al.*, 1999
Lysozyme	Hydrolyzes bacterial cell walls	Phi van, 1996
*PTGIS, prostaglandin synthase	Prostaglandin synthase	Yokoyama *et al.*, 1996
Transglutaminase	Forms isopeptide bonds	Mirza *et al.*, 1997
*Xanthine Oxidase	Oxidative metabolism of purines	Xu *et al.*, 1996
Miscellaneous		
alpha-1 acid glycoprotein	Serum protein	Mejdoubi *et al.*, 1999
Apolipoprotein C III	Apoprotein of HDL	Gruber *et al.*, 1994
*Biglycan	Connective tissue proteoglycan	Ungefroren and Krull, 1996
Cyclin D1	Cell-cycle regulation	Guttridge *et al.*, 1999; Hinz *et al.*, 1999
*Cyclin D3	Cell-cycle regulation	Wang *et al.*, 1996b
Factor VIII	Hemostasis	Figueiredo and Brownlee, 1995
Galectin 3	β-galactosidase-binding lectin	Hsu *et al.*, 1996
HMG14	High mobility group 14	Walker and Enrietto, 1996
K3 Keratin	Intermediate filament protein	Wu *et al.*, 1994
Laminin B2 Chain	Basement membrane protein	Richardson *et al.*, 1995
Mts1	Multiple tumor suppressor	Tulchinsky *et al.*, 1997
*Pax8	Paired box gene	Okladnova *et al.*, 1997
*UCP-2	Uncoupling protein-2	Lee *et al.*, 1999a
Vimentin	Intermediate filament protein	Lilienbaum *et al.*, 1990
Wilm s Tumor Supressor Gene	Tumor suppressor	Dehbi *et al.*, 1998
α1-antitrypsin	Protease inhibitor	Ray *et al.*, 1995

Where possible, the first publication to report the data is given as a reference. *Genes contain NF-κB binding sites in their promoter/enhancer regions, but further experiments are required to prove their functionality

by therapeutic drugs, including various chemotherapeutic agents (Table 1). One may speculate that the activators and the target genes of this multifunctional transcription factor have co-evolved. While environmental stresses and xenobiotics activate NF-κB, its target genes include many cell surface receptors, among them the *mdr-1* gene, which encodes the multiple drug resistance mediator. Likewise, while modified proteins such as advanced glycated end products (AGEs) induce NF-κB (Table 1), the AGE receptor (RAGE) is an NF-κB target gene (Table 2).

Another recently recognized cellular stress has been termed the ER-Overload Response (Pahl and Baeuerle, 1997a, 1997b; Pahl, 1999). ER-overload arises from an accumulation of proteins within the endoplasmic reticulum (ER). It can occur under a variety of circumstances:

(1) a sudden increase in the production of proteins which enter the ER, for example during viral infection;
(2) drugs which interfere with ER function thereby leading to protein accumulation in the organelle;
(3) production of mutant proteins, which cannot fold correctly and thus accumulate in the ER; and
(4) an overproduction of wild-type proteins, for example during transient transfection experiments, which overwhelm the ER folding/processing machinery and therefore also accumulate in the organelle (Table 1).

Agents eliciting the ER-Overload Response thus appear under various categories in Table 1.

Cellular stress can result in the most drastic form of cellular self defense, namely programmed cell death or apoptosis. It is now clear that NF-κB can exert both pro- and anti-apoptotic effects in different cells types (Barkett and Gilmore, 1999, this issue). The observation that several stimuli, among them tumor necrosis factor a (TNFα) and binding to the IgM receptor (or cross linking of the receptor with antibodies), can lead both to NF-κB activation (Table 1) and to apoptosis (Laster *et al.*, 1988; Hasbold and Klaus, 1990) suggested that NF-κB induction was pro-apoptotic. Furthermore, both cross-linking of the Fas-receptor by anti-Fas antibodies and binding of the Trail receptors 1 and 2 stimulate NF-κB (Table 1). Moreover, both the Fas-receptor and its ligand are encoded by NF-κB target genes (Table 2). However, cells derived from RelA knockout mice are more susceptible to apoptosis induced by various agents, including TNFα (Beg and Baltimore, 1996; Van Antwerp *et al.*, 1996; Wang *et al.*, 1996a). Likewise, binding of the Trail receptor-4 induces NF-κB but prevents Trail-mediated apoptosis (Degli-Esposti *et al.*, 1997), and inhibition of NF-κB restores drug-induced apoptosis sensitivity to certain drug-resistant primary leukemic cells and leukemic cell lines (Jeremias *et al.*, 1998). These data, together with the identification of pro-survival *bcl-2* homologs, Bfl1/A1, Bcl-x_L and Nr13, and inhibitors of apoptosis (IAPs) as NF-κB target genes (Table 2), suggest that NF-κB activation is anti-apoptotic in several cell types.

Consistent with a pro-surivival activity for NF-κB, several mitogens and growth factors stimulate NF-κB (Table 1) or are induced by NF-κB (Table 2). Some of these mitogens, such as M-CSF and PDGF, appear to act via an autocrine loop: they activate NF-κB which in turn stimulates transcription of the growth factor

gene. Because mitogens and growth factors stimulate NF-κB activity it is logical that several early response genes are also regulated by this transcription factor (Table 2). Thus, in addition to immune modulation and the more general stress response, NF-κB appears to promote cell survival. Teleologically speaking, it may 'make sense' to couple a stress response factor to anti-apoptotic pathways. This central coordinator evokes an effective response against the stress and ensures that the cell does not succumb in the process.

Many physiological mediators that bear no apparent connection to stress responses also activate NF-κB (Table 1). Among these mediators are several, such as PAF and Bradikinin, that activate NF-κB and whose receptors are NF-κB target genes (Table 2). Perhaps these mediators are released under conditions which have not been recognized or categorized as 'stress'. This, however, is only a question of definition.

Of the many chemical agents that induce NF-κB activity (Table 1), most elicit stress of some sort. Cycloheximide, for example, inhibits protein synthesis, while tunicamycin, brefeldin A, 2-deoxyglucose and monensin disrupt ER function, thereby eliciting ER-overload. Nocodazol, calchicine, podophyllotoxin and vinblastin interfere with microtubule function (Rosette and Karin, 1995). Systematic screening of chemical libraries would surely unearth a plethora of additional agents that induce NF-κB, perhaps by interfering with vital cell functions, thereby causing stress.

In addition to the response genes already discussed, NF-κB activation leads to the transcriptional induction of various transcription factor genes, some themselves members of the Rel/NF-κB/IκB family. In this way, NF-κB limits its own activation, in that NF-κB activation results in the new synthesis of its inhibitor IκB (Table 2). Newly-synthesized IκB can enter the nucleus and dislodge active NF-κB from its DNA binding site (Zabel and Baeuerle, 1990, Zabel *et al.*, 1993). Thus, in most cell types, NF-κB activation is transient. However, because NF-κB can induce the transcription of other transcription factors, for instance the proto-oncogene c-*myc* and the tumor suppressor *p53*, an initial NF-κB activation may indirectly induce the transcription of many more genes than the identified 150 targets.

Thus, a more detailed look at inducers and targets of NF-κB suggests that this transcription factor is more than a mediator of the immune response. It appears that NF-κB is activated and induces responses to various forms of cell stress and should therefore more generally be termed a 'central mediator of human stress response'. In this context it is interesting to note that certain well-studied stress situations, such as heat shock and the unfolded protein response, do not activate NF-κB. NF-κB activity, therefore, appears reserved for select but widely varied stresses.

New roles for NF-κB?

There are many NF-κB target genes whose properties and function defy classification (listed under Enzymes and Miscellaneous in Table 2). Several of these target genes, such as vimentin, laminin, collagenase and gelatinase, appear to involve NF-κB in the regulation of cell structure and micro-environment. Such an adaptation response may also serve to reduce cell stress, however, more data are required to evaluate a role for NF-κB in these processes. Likewise, the observations that NF-κB regulates transcription of the cyclin D1 gene (Guttridge *et al.*, 1999; Hinz *et al.*, 1999) and may also participate in cyclin D3 gene transcription (Wang *et al.*, 1996b) are very intriguing. If NF-κB controls cyclin transcription, this would implicate NF-κB in cell-cycle progression. However, more data are required to substantiate this idea.

Specificity of the NF-κB response

NF-κB participates in the transcription of over 150 target genes. Are all activated when NF-κB is induced? How can this transcription factor maintain any selectivity or specificity?

For NF-κB activation the selectivity resides mainly in the cell type targeted. Not all cell types respond equally to a given stimulus, either because they lack the cognate receptor or because they lack the required signal transduction molecules (discussed in Karin, 1999, this issue). Thus, not every stimulus listed in Table 1 will activate NF-κB in every cell type examined.

Several different mechanisms confer selectivity on the transcriptional response to NF-κB activation. These include:

(1) the combinatorial response of promoter/enhancer regions, and
(2) The selective activation and binding of individual Rel/NF-κB proteins.

The combinatorial response

The promoter/enhancer regions of most genes contain more than one transcription factor response element. Therefore, more than one transcription factor is usually required to induce effective transcription of a given gene. For the target genes listed in Table 2, NF-κB activity is necessary for efficient transcription. Thus, mutation of the κB site abrogates transcription of these promoters. However, NF-κB may not be sufficient for full transcription, as other transcription factors are also required. This combinatorial regulation of transcription provides specificity to a given response. While a given stimulus may activate NF-κB, if it fails to activate additional transcription factors, the target gene will not be transcribed fully.

We have demonstrated such selectivity using stably transfected cell lines, which express a chimeric p50/VP16 protein. In this fusion protein, the p50 DNA-binding domain confers specific binding to κB sites, while the HSV VP16 transactivation domain provides potent transcriptional activation. Expression of the p50/VP16 protein was placed under the control of a tetracycline repressable promoter in CMS-5 fibroblast cells. While induction of p50/VP16 was sufficient to activate transcription of the GM-CSF gene in these cells, transcription of the NF-κB target genes IL-1, IL-2 and IL-6 was not induced (Meerpohl and Pahl, unpublished observations). Thus, despite the almost overwhelming number of NF-κB target genes, the individual gene is selectively activated under specific circumstances.

Selective activation of Rel/NF-κB family members

The various members of the Rel/NF-κB family differ in their preference for specific DNA-binding sites (Kunsch *et al.*, 1992). Using random oligonucleotides and a DNA-binding assay, Kunsch *et al.* (1992) selected optimal DNA-binding sites for p50, RelA and c-Rel, as homodimers. The preferred binding site differs for each homodimer. Not surprisingly, such specificity is also seen *in vivo*. For example, several NF-κB target genes have been reported to contain binding sites that preferentially bound by RelA homodimers (e.g., ICAM-1 (Ledebur and Parks, 1995) and IL-4 (Casolaro *et al.*, 1995)).

Thus, the availability of different Rel/NF-κB hetero- and homodimers, whose synthesis and activation may be controlled by distinct signal transduction pathways, provides an additional level of selectivity in NF-κB-mediated gene transcription.

Demonstration of NF-κB activation and discovery of target genes

The simplest and most reliable assay to demonstrate NF-κB activation is the electrophoretic mobility shift assay (EMSA, (Müller *et al.*, 1997)). When combined with the appropriate controls, i.e. unstimulated control cells, as well as competition and supershift assays, the EMSA is both sensitive and accurate. Transient transfection experiments using a κB site-driven reporter gene are also frequently used. The appropriate controls include either a reporter gene lacking the κB sites, or, even better, a reporter gene preceded by mutated κB sites. Inhibition of reporter gene expression by co-transfection of an expression vector for the NF-κB inhibitor IκB provides an additional confirmation of specificity. All of the inducers listed in Table 1 have been demonstrated to activate NF-κB using one of these assays.

What makes a gene an NF-κB target gene? Not all genes listed in Table 2 are *bona fide* NF-κB target genes. That is, genes in Table 2 preceded by an asterisk merely contain a putative NF-κB binding site in their promoter/enhancer region. To establish that a gene is truly regulated by NF-κB, the following experiments must be performed:

(1) NF-κB binding to the putative DNA site must be shown in an EMSA, preferably using cell extracts from a tissue which usually expresses the gene under investigation, and

(2) the promoter/enhancer region must be cloned in front of a reporter gene and functional importance of the κB site must be demonstrated by mutagenesis.

These two experiments are required before a gene can be considered an NF-κB target. However, these data can be misleading if, for example, NF-κB can bind to a DNA sequence, but this site or adjacent sequences are occupied by other proteins *in vivo*. In such a scenario, NF-κB would bind the isolated site *in vitro* in an EMSA. Mutation of this site would result in a loss of function in reporter gene assays, but only because binding of the adjacent unidentified protein is lost. Therefore, for final proof of NF-κB involvement, *in vivo* DNA footprinting of the κB site should be performed. This, however, has not been done for the majority of the target genes listed in Table 2. Nevertheless, the vast majority of these genes are expected to be true NF-κB target genes. Moreover, it is likely that cDNA microarray technologies and genomic sequencing will identify many other NF-κB target genes.

Summary

This article lists and categorizes the known inducers and target genes of the pleiotropic transcription factor NF-κB known to date. Compilation of these data reveals that the vast majority of NF-κB inducing agents or conditions represent a form of stress to cells. In response, many NF-κB target genes function to alleviate cell stress. In addition, NF-κB has recently been shown to inhibit apoptosis in several cell types. Therefore, NF-κB may act as a central integrator of stress responses and cell survival pathways. The rapid rate at which new NF-κB inducers and target genes are being identified suggests that this transcription factor may coordinate additional cellular functions.

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